# **PCT**

# WORD DAS TELLIAVITAL PROPERTY OF GANGES FION Enternational Eurean



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#### 57 Abstract

The consent invention of wide model inducated forms of vascular endethelial growth factor related piete ins NRPs or VRPs) which are set in the stimulation of announces is mention in or only. The invention also provides nucleic across encoding such notice, numerically the artificial producing to mented NRPs, pharmical compositions comprising translated NPPs and methods of gene therapy many the inches across which code for translation NRPs may be useful for the treatment of heart disease and for wound healing.

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#### DESCRIPTION

#### TRUNCATED VEGE-RELATED PROTEINS

## Field of The Invention

The present invention relates to novel truncated forms of vascular endothelial growth factor (VEGF)-related proteins. More particularly, the invention relates to N-terminally truncated VEGF-related proteins that are substantially free of other proteins. Such truncated VEGF-related proteins may be used to stimulate angiogenesis in vivo and in vitro.

The invention also relates to nucleic acids encoding such novel truncated VEGF-related proteins, cells, tissues and animals containing such nucleic acids; methods of treatment using such nucleic acids; and methods relating to all of the foregoing.

#### Background

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Vascular endothelial growth factors (VEGFs), also called vascular permeability factors (VPFs:, are a family of proteins that are produced by many different cell types in many organs and act in a highly selective manner to stimulate endothelial cells almost exclusively (reviewed in Ferrara et al., Endocr. Rev. 13:18-32, (1992); Dvorak et al., Am. J. Pathol. 146:1029-39, 1995; Thomas, J. Biol. Chem. 271:603-06, 1996). These publications, and all other publications referenced herein, are hereby incorporated by reference in their entirety.

When tested in dell dulture, VEGFs are potently mitogenia to approximately at al., <u>Prov. Math. Acad. Sci. USA</u> 96:7311-15, 17677 and onemotiants 'Favard at al., <u>Prol. Mell</u> 73:1-6, 13314. Availthinally, VEGFs induse plasminoden activator, plasminoder activator inhibitor, and plasminogen activator receptor (Manariota et al., <u>F. Biol. Chem. 200:8719-16, 1995; Popper et al., 161: 300-06, 19917, as well as collagenases (Unemori et al., J. Bell. light. 133:377-61, 1992), onzyma systems that</u>

also stimulate the formation of tube-like structures by endotnelial cells, an in vitro example of angiogenesis (Nicosia et al., Am. J. Pathol., 145:1023-29, 1994).

In vivi, VEGFs induce angiogenesis (Leung et al., Science 246:1306-09, 1989) and increase vascular permeability (Senger et al., <u>Science</u> 219:983-85, 1983). VEGEs are now known as important physiological regulators of capillary blood vessel formation. They are involved in the normal formation of new capillaries during organ growth, including fetal growth (Peters 10 et al., Proc. Natl. Acad. Sci. USA 93:8915-19, 1993), tissue repair (Brown et al., <u>J. Exp. Med.</u> 176:1375-79, 1992), the menstrual cycle, and pregnancy (Jackson et al., Placenta 15:341-53, 1994; Cullinan & Koos. Endocrinology 133:839-37. 1993; Kamat et al., Am. J. Pathol. 146:157-65, 1995). During 15 fetal development, VEGFs appear to play an essential role in the de novo formation of blood vessels from blood islands (Risau & Flamme, Ann. Rev. Cell. Dev. Bicl. 11:73-92, 1995), as evidenced by abnormal blocd vessel development and lethality in embryos lacking a single VEGF allele (Carmeliet et al., Nature 20 380:435-38, 1996). Moreover, VEGFs are strongly implicated in the pathological blood vessel growth characteristic of many diseases, including solid tumors (Potgens et al., Biol. Chem. Hoppe-Seyler 376:57-70, 1995), retinopathies (Miller et al., Am. J. Fathol. 145:574-84, 1994; Aiello et al., N. Engl. J. Med. 331:1480-87, 1994; Adamis et al., Am. J. Ophthalmol. 25 118:445-50, 1994), pscriasis (Detmar et al., J. Exp. Med. 180:1141-46, 1994', and rheumatoid arthritis (Fava et al., J. Exp. Man. 1:0:341-40, 1894 .

VEST expression is regulated by strminer dishweiki et al., 1. 1116. Invest. 91:2111-12, 1995; priving factors Thomas, 2. Biol. Chem. 271:603-06, 1996), and by sypoxia (Schweiki et al., Nature 359:843-45, 1992, Levy et al., 3. Biol. Chem. 271:2746-53, 1996). Opresulation if VESEs by hypoxic conditions is of particular importance as a temperature medianism for which

tissues increase oxygenation through industion of additional dapillary vessel formation and resulting increased blood flow. This mechanism is thought to contribute to pathological anguagenesis in tumors and in retinopathies. However, upregulation of VEGF expression after hypoxia is also essential in tissue repair, e.g., in dermal wound healing (Frank et al., J. Biol. Chem. 270:12607-613, 1995), and in coronary ischemia (Banai et al., Cardiovaso, Res. 28:1176-79, 1994; Hashimoto et al., Am. J. Physicl. 267:H1948-H1954, 1994).

The potential of VEGF to pharmacologically induce 10 anglogenesis in animal models of vascular ischemia has been shown in the rabbit chronic limb ischemia model by cemonstrating that repeated intramuscular injection or a single intra-arterial bolus of VEGF can augment collateral blood 15 vessel formation as evidenced by blood flow measurement in the ischemic hindlimb (Pu, et al., Circulation 88:208-18, 1993; Bauters et al., Am. J. Physiol. 267:H1263-71, 1994; Takeshita et al., <u>Circulation</u> 90 [part 2], II-223-34, 1994; Bauters et al., <u>J. Vasc. Surg.</u> 21:314-25, 1995; Bauters et al., Circulation 31:2802- 09, 1995; Takeshita et al., J. Clin. 20 Invest. 93:662-70, 1994). In this model, VEGF has also been shown to act synergistically with basic FGF to ameliorate ischemia (Asahara et al., Circulation 92:[suppl 2], II-365-71, 1995). VEGF was also reported to accelerate the repair of balloon-injured rat carotid artery endothelium thereby inhibiting pathological thickening of the underlying smooth mussle layers, and thus maintaining lumen diameter and plood plow Asanada -- al., directation 01:0743-2801, 19954. VEGE has also reen shown to induce EDRF (Indotnelium-Jesived feraming Factor (nitric oxide,) edependent relaxation in Janine porgnary arteries, thus potentially contributing to increased blood flow to ischemic areas via a secondary mechanism not Felato: to angiquenesis. To obtail, Am. J. Enverol.  $265:958\ell-$ Hit. 1997 Together, tress that arrived a compatible set spidence

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for a potential therapeutic role of VEGFs in wound healing, ischemic diseases and restenosis.

The VEGF family of proteins is comprised of at least 4members VEGF-121, VEGF-165, VEGF-189, and VEGF-206. brighnally characterized VEGF is a 34-45 kDa glycosylated protein which consists of 2 identical subunits of 165 amino abid residues (Tischer et al., Biochem. Biophys. Res. Commun. 165:1198-1206, 1989). The VEGF-165 cDNA encodes a 191-residue amino acid sequence consisting of a 26-residue secretory signal 10 peptide sequence, which is cleaved upon secretion of the protein from cells, and the 165-residue mature protein subunit. VEGF-165 binds strongly to heparin for which the strongly basic sequence between residues 115-159 is thought to be responsible (Fig. 1) (Thomas, J. Biol. Cnem., 271:603-06 (1996)). 15 other members of the VEGF family are homodimeric proteins with shorter or longer subunits of 121, 189 and 206 residues (VEGF-121, VEGF-189, and VEGF-206, respectively) (Tischer et al., J. Biol. Chem. 266:11947-54, 1991; Park et al., Mol Biol Cell 4:1317-26 (1993)). The 4 forms of VEGF arise from alternative 20 splicing of up to 8 exons of the VEGF gene (VEGF-121, exons 1-5,3; VEGF-165, exons 1-5,7,8; VEGF-189, exons 1-5, &a, 7, 8; VEGF-206, expns 1-5, 6b, 7, 8 (excn 6a and 6b refer to 2 alternatively spliced forms of the same exon)) (Houck et al., Mol. Endocr., 5:1806-14 (1991)). The VEGF sequences contain 25 eight conserved disulfide-forming core cysteine residues. All VEGF genes encode signal peptides that direct the protein into the secretory pathway. However, only VEGF-121 and -165 are 1 Mult to be recally serreted by cultured calls whereas VESF-149 and -1 ( commany and silves with the extracollular matrix. These VESE time prosess to Additional Righty assis storings. corresponding to residues 110-139 in VEGF-189 and -236 (matrix-"argeting sequence,, which confers high affinity to acidic components of the extracellular matrix Thomas, 7. Biel. Chem. . P.: 6-1-00 (1396 ).

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Mitogenia activity of the various VEGF isoforms varies depending on each isoform. For example, VEGF-121 and VEGF-165 have very similar mitogenia activity for endothelial cells. However, VEGF-183 and VEGF-206 are only weakly mitogenia (Ferrara et al., Endoor, Rev. 13:18-32, 1992). The reduced activity of these isoforms is attributed to their strong association with cells and matrix, as evidenced by the normal mitogenia activity of a mutant of VEGF-206 which lacks the 24-residue "matrix targeting" sequence common to VEGF-183 and VEGF-206 (residues 115-139 in Fig. 1) (Ferrara et al., Endoor, Rev. 13:18-32, 1992).

An N-terminal fragment of VEGF-165 generated by plasmin (VFGF (1-111)) bound with the same affinity to the KDR receptor as VEGF-165 and VEGF-121 whereas the C-terminal VEGF-fragment (111-165) had no binding activity (Keyt et al., J. Biol. Chem. 271:7788-95, 1996). Interestingly, in this study the mitogenic activity of VEGF-121 and VEGF-110 was reduced by approximately 110-fold as compared to VEGF-165, suggesting a potential role of the C-terminal domain of VEGF-165 in the biological potency of VEGF isoforms. The significance of this finding is somewhat unclear in view of earlier results showing the equivalent potency of VEGF-121 and VEGF-165 on endothelial cell growth. Furthermore, since functional interaction of VEGF with the KDR receptor is thought to be dependent at least in part on cell surface heparin sulfate proteoglycan(s) (Cohen et al., J. Biol. Chem., 270:11322-26, 1995; Tessler et al., <u>J. Biol. Chem.</u> 169:12436-61; 1994, it is conceivable that differences in recults arise from differences in various experimental systems. In this context in it includes to white expent cell surface reparts bulbases regulate the cunttional interaction of VESF-121 (lacking a heparin-binding domain) and VEGF-165 (possessing a heparin-pinding domain: (Tessler et al., J. Biol. Chem. 2-7:12456-61, 1:04; Roman et Wil, 1. Bibl. Chem. 270:11322-36, 1999: Bursh-Birsh Angli, J. Burt, W.W. Ongari, Again Gabi

VEGFs are related to platelet-derived growth factor (PDGF) [Andersson et al., Growth Factors 12:159-64, 1995). MEGFs are also related to the family of proteins derived from the Placenta Growth Factor (PIGF) gene, PIGF-129 and PIGF-150 (Maglione et al., Proc. Natl. Acad. Sci. USA 88:9267-71, 1991; Onaccene 8:925-31, 1993). More recently several additional VEGF-related genes have been identified and termed VEGF-B (also called VEGF-related factor VRF-1) (Grimmond et al., Genome Res. 6:122-29, 1396; Olofsson et al., Proc. Natl. Acad. Sci. U.S.A. 93:2567-31, 1996) VRF-2 (Grimmond et al., Genome Res. 6:122-29, 10 1996), and VEGF-C (Joukov et al., <u>EMBO J.</u> 15:290-98, 1996; Lee et al., Proc. Natl. Acad. Sci. USA 93:1989-92, 1996) and VEGF-3 FFCT Application No. PCT/US95/07283, published on December 12. 1996 as WO96/39421). Finally, two virally encoded VEGF-related 15 sequences have been identified, poxvirus CRF-1 and ORF-2 (Lyttle et al., J. Virol. 68:84-92, 1994). With the exception of PDGF, these proteins are referred to as VEGF-related proteins [VRPs]. Sequences of examples of VRPs are depicted ir. Figure 1.

The VRPs, and the PDGFs known so far have 8 cysteines within their sequences that are relatively positionally conserved. The protein sequence spanning the conserved cysteines is therefore referred to herein as the core sequence, and the first N-terminal conserved cysteine of the sequence is referred to herein as the "First cysteine of the core sequence" or "first core cysteine."

Interestingly, members of the VEGF families can form deteridimens, such as neterodimens discipling to VEGF and EIGF signature (Didalvo et al., J. Biol. Them. 270: 7017-12, 1906; Dan et al., J. Book. The Biol. Them. 270: 7017-12, 1906; Dan hadnly potent in stimulating and openesis and endothelial cell proliferation, VEGF/PIGF neterodimens are less potent mitogens, and PIGF compaiment have little from mitogenic activity (Didalvo et al., J. Bjj., Chem. 200: 7000-23, 190: Tan et al.,

1. Figl. Chem. 271: 3154-62, 1996). In other experiments, VESF-165/VESF-8 heterodimers were found to form after transfection of cells with both genes (Olofsson et al., Froc. Matl. Acad. Sci. U.S.A. 93:2567-81, 1996).

VEGFs interact with two receptors present on endothelial cells, KDR/flk-1 (Terman et al., <u>Biochem. Biophys. Res. Commun.</u> 187:1579-86, 1992), and flt-1 (De Vries et al., <u>Science</u> 255:989-91, 1992). Systematic site-directed mutagenesis of VEGF-165 by alanine scanning of charged residues has shown that residues D63, E64 and E67 are involved in binding of VEGF to flt-1 whereas the basic residues R82, KI84, and H86 contribute strongly to binding to KDR (Keyt et al., <u>J. Biol. Chem.</u> 271:5638-46, 1996).

VRPs are known to bind to one or more of three different endothelial cell receptors, each of which is a single transmemorane protein with a large extracellular portion comprised of 7 immunoglobulin-type domains and a cytoplasmic portion that functions as a tyrosine kinase. These receptors are KDR/flk-1 (Terman et al., Biochem. Biophys. Res. Commun. 187:1579-86, 1992), flt-1 (De Vries et al., Science 258:989-91, 1992), and flt-4 (Pajusola et al., <u>Cancer Res.</u> 52:5738-43, 1992). There are distinct selectivities between these receptors and the various VEGF ligands that have not been completely elucidated as yet. However, it is known that VEGF 23 binds to KDR and fltl (Terman et al., Growth Factors 11:137-35, 1994) but not flt4 (Joukov et al., <u>EMBO J.</u> 15:290-99, 1996), PIGF binds to fit I but not KDR (Terman et al., Growth Factore 1::1- --4, 1994 and fl:4 - Jouksy et al., EMRO J. 16:030--4. 1996 , VEGF-T pinds to fit-4 - Trukov et al., EMBO 7. 18:090-94, 1990; Pub It to controversial whether it also block to MIP (Joukhy et al., EMBC U. 15:290-98, 1996; Lee et al., <u>Proc.</u> Natl. Acad. Sci. USA 93:1988-92, 1996). The receptor specificity for VEGE-B/VFE-1, VPE-D and the virgily encoded WREK I not prosently known. However, since VRSF-8 stimulates

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endothelial cell proliferation (Clofsson et al., <u>Proc. Natl. Acad. Sci. U.S.A.</u> 93:2567-81, 1996) it may be speculated that VEGF-B can bind to KDE because KIR is thought to be primarily responsible for the angiogenic response of endothelial cells to VEGF-like growth factors (Gitay-Coren et al., <u>J. Biol. Chem.</u> 271:5519-23 (1996)).

Most of the VRPs have been shown to activate the KDR receptor which is thought to make endothelial cells "angicgenesis-competent." Evidence for such activity has been presented for VEGF-B which stimulates endothelial cell proliferation (Olofsson et al., Free. Natl. Acad. Sci. U.S.A. 93:2567-81, 1996), VEGF-C which stimulates endothelial cell migration and proliferation (Joukev et al., EMBO J. 15:290-98, 1996; Lee et al., Proc. Natl. Acad. Sci. USA 93:1988-92, 1996), and both known virally encoded VFPs which were reported to be angiogenic (Lyttle et al., C. Virol. 68:34-92, 1994). A notable exception are PIGF isoform homodimers which have negligible mitogenic activity for endothelial cells. However, PIGF/VEGF heterodimers still retain considerable mitogenic activity (DiSalvo et al., J. Biol. Chem. 270:7717-23, 1995; Cao et al., J. Biol. Chem. 271: 3154-62, 1996).

VEGFs are expressed in many different tissues. Similarly, VRP genes are also expressed in multiple tissues but it is of particular interest that VEGF-B and to a lesser extent VRF-2 are strongly expressed in human heart and skeletal mustle (Grimmond et al., Genome Res. 6:122-29, 1996; Olofsson et al., Erg.: Natl. Anal. Sci. U.S.A. 93:2567-91, 1996). In fact, VEGF-B is expressed considerably more strongly in mouse heart tissue than VEGF . Diofsson et al., 1995. Natl. Anal. Aci. 1998. Page 1998. This several human tissues, most notably in heart and skeletal muscle (Joukov et al., EMBO J. 15:290-98, 1996). This expressed in factors of factors, as for expression appoints by a factor of the page 1998.

physiological role in angiogenesis in these tissues. This is thought to be relevant in pathological situations such as coronary isonemia where collateral angiogenesis is required to provide the heart muscle with an adequate capillary blood vessel supply. It has been shown that transient ischemia induced by coronary artery ligation or hypoxia rapidly upregulates VEGF mRNA in the rat or pig heart in vivo and hypoxia induces VEGF mRNA in cardiac myocytes and smooth muscle cells in witro (Hashimoto et al., Am. J. Physiol 267, H1948-H1954, 1994; Banai, et al., Cardiovac. Res. 28:1176-79, 1994; Circulation 90, 649-52, 1994). The strong expression of VEGF and VRPs in the heart may help to ensure a redundant and competent regulatory system capable of inducing new blood vessel formation when it is needed. Collateral blood vessel 15 formation is also required in peripheral (lower limb) vascular ischemias and in cerebral ischemias (stroke). Finally, new blood vessel formation is required in tissue repair after wounding. In this context, it is worth noting that VEGF is upregulated in epidermal keratinocytes during skin wound 20 healing (Brown et al., J. Exp. Med. 176:1375-79, 1992). Thus, therapy of various ischemic conditions such as cardiac infarction, chronic coronary ischemia, chronic lower limb ischemia, wound healing and stroke with VRPs may be potentially clinically beneficial.

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## Summary Of The Invention

The present invention is directed to novel truncated forms of VEGF-related proteins VFPs', preferably numan VRPs. The preferred use of the truncated VFFs and nucloid acid melocile mapositions of the invention of a week size commonstions to aid in the treatment of patients with heart disease, wounds, or other isonemic conditions by stimulating anglogenesis in such patients. The amint sold requences of VFPs include sight disulfide-forming systems are that are a majoryes for week

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VRFs and VEGF proteins (core cysteines). VRPs include, but are not limited to, VEGF-B, VEGF-C, VRF-1, ORF-1, ORF-2, and PIGFs.

A first aspect of the invention provides for a truncated VRP naving a deletion of at least one of the amino acid residues N-terminal to the first cysteine of the core sequence of said subunit. Such compositions would be substantially free of other proteins. Freferably, the truncations range from truncating minimally the N-terminal residue of the mature protein subunit only(not including the signal sequence) and maximally all N-terminal amino acids of the mature protein up to and including the residue N-terminal to (prior to) the first core cysteine residue. In more preferred aspects, all of the amino acid residues N-terminal to the first cysteine of the core sequence, except the 1 to 5 amino acid residues immediately N-terminal to said first cysteine, are deleted.

Although the amino acid deletions may consist of deletions of non-adjacent amino acid residues in the N-terminal sequence, it is preferred that the deletions be of consecutive amino acid residues. Thus, in one preferred aspect, the invention comprises human VRPs that have deletions of amino acid residue sequences of increasing lengths from the N-terminus of the N-terminal sequence up to the first cysteine of the core sequence of the VRP subunit sequence.

In preferred aspects, the invention provides for truncated versions of the VRPs VEGF-B, VRF-2, VEGF-C, VEGF-3, PlGF, poxvirus ORF-1, and poxvirus ORF-2. In such truncated VRPs, each subunit may independently have a deletion of at least one of the amino acid residues N-terminal to the cirst dysteine of the cors sequence of said subunit, or only one of the subunits may have such a deletion.

In particular emediments, the truncated VRP subunit comprises a VRP subunit wherein various numbers of amino acid reclaus N-terminal to the first systems if the core requence are 14 ated of the core requence.

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consist of consecutive amino acid residues derived from the N-terminal sequence. These consecutive N-terminal residues may be derived from any location in the N-terminal sequence, however, a consecutive sequence starting from the N-terminus of the N-terminal sequence is preferred, and a sequence consisting of consecutive amino acid residues immediately N-terminal to the first cysteine of the core sequence of the VRP subunit is most preferred. Examples of such most preferred embodiments are depicted in Figure 2.

In other embodiments, the amino acid residues N-terminal to the first cysteine of the core sequence of the truncated VRPs of the invention are a randomly selected amino acid sequence, in yet other embodiments, these amino acid residues are derived from the N-terminal sequence of the full length VRP sequence, but are not necessarily consecutive amino acids from the full length VRP sequence.

Thus, in one most preferred aspect, the invention provides a truncated VRP subunit wherein the amino acid residues N-terminal to the first cysteine of the core sequence of said subunit are deleted.

In other aspects, the invention provides a truncated VRP subunit wherein the amino acid sequence N-terminal to the core sequence comprises 11 to 20, more preferably 11 to 15, more preferably 6 to 10, and most preferably 2 to 5 amino acid residues

Preferably, the amino acid sequence N-terminal to the core squence comprises the consecutive amino acid residues immediately N-terminal to the first cysteine of the core sequence of said VRI subunit. Thus, in these preferred onto simonts, this truncation VRP comprises the size sequence, the necessary C-terminal sequence to the core sequence, and further comprises at the region N-terminal to the first cysteine of the top comprises at the region N-terminal to the first cysteine of the top comprises at the region N-terminal to the first cysteine of the top comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprises at the region N-terminal to the first cysteine of the comprise of th

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consecutive amino acid residues of the amino acid sequence that is immediately N-terminal to the first cysteine of the core sequence of the full length VRP sequence.

Those skilled in the art will recognize that where a truncated VRP subunit comprises, for example, (X) amino acids N-terminal to the first cysteine of the core sequence, that such a truncated VRP subunit is one where the corresponding full length VRP subunit comprises (X+1) amino acids N-terminal to the first cysteine of the core sequence.

The truncated VRPs of the invention include truncated VRP homodimers comprising two truncated VRP subunits of the invention, wherein the two truncated VRP subunits have the same amino acid sequence, and also include truncated VRP heterodimers comprising two truncated VRP subunits of the invention wherein the two subunits have different amino acid sequences from each other.

For purposes of the present invention, the term "first N-NN" amino acids where N and NN each represent numbers of amino acids, for example, the first 10-15 amino acids, denotes the first N-NN amino acids (e.g., the first 10-15 amino acids) after the signal peptide sequence of the designated VRP. The term N-NN encompasses a deletion of anywhere from N to NN of the first amino acids after the signal sequence. Thus, in more preferred aspects, the truncated VRP subunit comprises a truncated hVEGFB protein subunit wherein the first 16-15 amino acids are deleted; more preferably, the first 15-20 amino acids are deleted; more preferably, the first 16-23 amino acids are deleted; note preferably, the first 16-23 amino acids are deleted; and many preferably, the first 16-23 amino acids are deleted; and many preferably, the first 16-23 amino acids are deleted; and many preferably, the first 16-23 amino acids are deleted; and many preferably, the first 16-23 amino acids are deleted.

In their sire preferrer aspects, the transited VPF dibinit comprises a truncater sVRF2 protein subunit wherein the first 10-15 amino acids are deleted; more preferably, the first 15-20 amino acids are deleted; more preferably, the first 10-21 amino

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acids are deleted; and most preferably, the first 13-24 amino acids are deleted.

In other more preferred aspects, the truncated VRF subunit comprises a truncated hVEGFC protein subunit wherein the first 95-100 amino acids are deleted; more preferably, the first 100-105 amino acids are deleted; more preferably, the first 105-110 amino acids are deleted; and most preferably, the first 108-109 amino acids are deleted.

In other more preferred aspects, the truncated VRP subunit comprises a truncated hPIGF protein subunit wherein the first 15-21 amino acids are deleted; more preferably, the first 21-26 amino acids are deleted; more preferably, the first 26-31 amino acids are deleted; and most preferably, the first 29-30 amino acids are deleted.

In other more preferred aspects, the truncated VRP subunit comprises a truncated hVEGF3 protein subunit wherein the first 10-15 amino acids are deleted; more preferably, the first 15-20 amino acids are deleted; more preferably, the first 20-25 amino acids are deleted; and most preferably, the first 23-24 amino acids are deleted.

In other more preferred aspects, the truncated VRP subunit comprises a truncated pvCRF1 protein subunit wherein the first 20-25 amino acids are deleted; more preferably, the first 25-30 amino acids are deleted; more preferably, the first 30-35 amino acids are deleted; and most preferably, the first 33-34 amino acids are deleted.

In other more preferred aspects, the truncated VRP subunit comprises a truncated pyCRF1 protein subunit wherein the first 30-30 mmin, using are deleted; more preferably, the first 30-40 amin's egide are deleted; more preferably, the first 40-45 amin acids are deleted; and most preferably, the first 43-44 amino acids are deleted. The sequences of some exemplary preferred truncated VRP subunits are set out in Figure 2.

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The invention also provides for nucleic acid molecules coding for the truncated VRP subunits described herein. The nubleto acid molecules may be, for example, DNA, cDNA or RNA. The invention also provides for recompinant DNA vectors comprising the nucleic acid molecules encoding the truncated VRPs, and host cells transformed with such recombinant DNA vectors, wherein such vectors direct the synthesis of a trunsated VRP subunit such as those described herein.

The invention further provides for nucleic acid molecules encoding bidsynthetic precursor forms of N-terminally truncated supunits of VRPs for the purpose of facilitating the expression in suitable host systems. Such nucleic acid molecules are comprised of DNA encoding a signal peptide that precedes the truncated subunits at their N-termini. The signal sequences of 15 VEGF or VRPs would be used to construct appropriate signal peptide-containing truncated forms of VRPs. The human VEGF signal peptide is as follows:

mnfllswyhwslalllylhhakwsqa I) -- [SEQ I.D. NO. 40] --Alternatively, the signal peptides shown in Figure 1 may be Preferably, the signal peptide specific for truncated VRP is used in the construct.

In order to facilitate signal peptide cleavage in mammalian cells after fusion of the signal sequence to truncated forms of VRP, it may be necessary to include the first or the first two residues of the mature MRP peptide sequence, e.g. proline (P), or proline-valine (PV) for hVEGFB. Thus, an appropriate nucleif abid molecule would be comprised of DNA encoding the signal dequence of VEGF-B, optionally followed by a godge for grolling (the first residue of mature VEGF-A , sectionally followed by a todom for viline (the second residue of mature VEGF-B), and followed by DNA encoding the Nterminally truncated VEGF-B. The invention also provides for ither appropriate signal poptine fision constructs, best idinapit  $\sigma \sim \pi$  memmelian none, an annum no inige salled in

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the art. Those skilled in the art will recognize that the signal peptides should optionally include residues needed for facilitation of signal peptide cleavage in mammalian cells for the various truncated VRP subunits of the present invention.

Thus, the present invention provides for recombinant DNA expression vectors wherein the 5' end of the nucleic acid molecule coding for the truncated VRP subunit is operably linked to a DNA sequence that codes for a signal peptide. signal peptide may be a human VRP signal peptide. Moreover, the DNA sequence coding for said signal peptide may be operably linked at the 3' end of said DNA sequence to DNA coding for the first amino acid residue of the mature non-truncated VRP subunit, and wherein the 3' end of said DNA coding for said residue is operably linked to the nucleic acid molecule coding for said truncated VRF subunit. In other aspects, the DNA sequence coding for said signal peptide is operably linked at the 3' end of said DNA sequence to DNA coding for the first two amino acid residues of the mature non-truncated VRP subunits, and wherein the 3' end of said DNA coding for said two residues is operably linked to said nucleic acid molecule coding for said truncated VRP subunit. Thus, in preferred aspects, the invention also provides a truncated VRP subunit of invention as described above, further communising at the Nterminus of said truncated VRP subunit, the first one or two amino acid residues of the mature non-truncated VRP subunit. Those skilled in the art will recognize that such truncated VRP subunits of the invention include those wherein the final number in amino ablie determinal to the first overeine of the Tore beginns finding the affiliantal one or two amino asids Andt may facilitat, filmal paptidi destage is at least one less than the number of amino acids N-terminal to the first cysteine of the core sequence of the corresponding full length WES.

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In other preferred aspects, the invention provides truncated VRP homodimers or neterodimers comprising two truncated VRP subunits wherein said truncated VRP subunits comprise at the N-terminus of said truncated VRP subunits, the first one or two amino acid residues of the mature non-truncated VRP subunit.

In preferred aspects, the recombinant nucleic acid molecule coding for a truncated VRP subunit of the invention is operably linked to control sequences operable in a host cell transformed with said vector. The present invention also provides for transformed or transfected host cells comprising the recombinant ENA vectors of the invention.

The present invention also includes delivery vectors which comprise nucleic acid molecules coding for the truncated VRPs of the invention. Such delivery vectors may be, for example, viral vectors. Such viral vectors may be, for example, adenovirus vectors or adenovirus-associated virus vectors. In other aspects of the invention are provided an adenovirus vector comprising a nucleic acid molecule coding for a truncated VRP of the invention operably linked at the 5' end of the nucleic acid molecule to a DNA sequence that codes for a signal peptide. Preferably, the signal peptide is selected from the group consisting of VEGF signal peptide, VEGF-B signal peptide, VRF-2 signal peptide, VEGF-C signal peptide, FlGF signal peptide, VEGF-3 signal peptide, poxvirus CRF-1 signal peptide, and poxvirus ORF-2 signal peptide. Preferably said ut mal bestide is MEGF-B signal peptial. In preferred aspects, the DNA sequence roding for the signal poptice is operably linked at the 3' and do the SNA sequence to BNA goding for the first amin. Isld residue of the matare nin-truncated /RP subunit, and wherein the ?' end of said ENA coding for said residue is operably linked to the nucleic acid molecule coding for this trunchton VRFs. In most preferred aspects, the

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adenovirus vector comprises a nucleic acid molecule which codes for a truncated VRP subunit of Figure 2.

In further preferred aspects of the invention are provided a filtered-injectable adenovirus vector preparation comprising a recombinant adenoviral vector, said vector containing no wild-type virus and comprising: a partial adenoviral sequence from which the ElA/ElB genes have been defeted, and a transgene coding for a truncated VEP subunit, driven by a promoter flanked by the partial adonovirus sequence; and 10 pharmaceutically acceptable carrier. In preferred aspects, the preparation has been filtered through a 30 micron filter. In other preferred aspects the truncated VEGF supunit is a truncated VEGF subunit of Figure 2. In another preferred aspect, the injectable adenoviral vector preparation comprises a promoter selected from the group consisting of a CMV promoter, a ventricular myocyte-specific promoter, and a myosin heavy chain promoter.

In other aspects, the invention provides a method of producing a truncated VRP polypeptide comprising growing, under suitable conditions, a host cell transformed or transfected with the recombinant DNA expression vector of the invention in a manner allowing expression of said polypeptide, and isolating said polypeptide from the host cell. Suitable conditions are then provided for the truncated VRP peptide to fold into a 25 truncated VRP subunit. In mammalian cells, such conditions should be naturally provided by the cell. In non-mammalian cells, appropriate pH, isotimicity, and feducing conditions much be provided, such as those destribed in, for example, Example 3. Main precensory, the inventity provides a method of production a truncated MRF whereout coutable conditions are provided for said truncated VPP supunit to dimerice with a second MRP subunit selected from the group consisting of MRP subunita and truncated URS subunita. In preferred aspects of Marinventina are provide; beginned in producing a truncated TRF

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homodimer comprising two truncated VRP subunits having the same amino acid sequence.

In other aspects of the invention are provided methods of producing truncated VRP heterodimers wherein the two VRP subunits have different amino acid sequences. Such heterodimers may consist of one truncated VRP subunit and one non-truncated VRP subunit, or both VRP subunits may be truncated. The two subunits may be derived from different VRPs. For example, the heterodimer may consist of one VEGF-B subunit and one truncated VEGF-C subunit, or both subunits may be truncated.

In further preferred aspects, the present invention provides pharmaceutical compositions comprising a truncated VFP subunit of the present invention, in a suitable carrier. The invention includes methods of stimulating blood vessel formation comprising administering to a patient such a pharmaceutical composition.

Methods are provided using the compounds of the present invention to stimulate endothelial cell growth or endothelial cell migration in vitro comprising treating said endothelial cells with truncated VRPs.

The present invention also provides methods of treating a patient suffering from a heart disease comprising administering to said patient a nucleic acid molecule coding for at least one truncated VRP subunit, said nucleic acid molecule capable of expressing the truncated VRP subunit in said patient. In an additional embodiment, methods are provided of stimulating analyzenesis in a patient comprising administering a therapeutic amount of a charmaceutical sumposition comprising a truncated VRP of the present invention.

Preferably, the pharmaceutical composition is in a therapeutically suitable delivery system. In other preferred aspects, a potentiating event is administered to potentiate the easilyment extent to go organizated VV... John adents include,

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for example, basic Fibroblast Growth Factor (bFGF) (FGF-2), acidic FGF (aFGF) (FGF-1), FGF-4, FGF-5, FGF-6, or any FGF or other angiogenic factor that stimulates endothelial cells. Thus, in one aspect of the invention is provided a pharmaceutical composition comprising a truncated VRP and one or more potentiating agents. The pharmaceutical compositions may also be used to treat patients suffering from ischemic conditions such as cardiac infarction, chronic coronary ischemia, chronic lower limb ischemia, stroke, and peripheral vascular disease. Methods are also provided using the pharmaceutical compositions of the present invention to treat wounds, such as dermal or intestinal wounds.

In preferred embodiments, methods are provided of stimulating angiogenesis in a patient comprising delivering a delivery vector to the myocardium of the patient by intracoronary injection directly into one or both coronary arteries, said vector comprising a nucleic acid molecule coding for at least one truncated VRP subunit, wherein said vector is capable of expressing the truncated VRP subunit in the myocardium.

In other preferred embodiments, the method may be used for stimulating coronary collateral vessel development.

In more preferred embodiments, a method is provided for stimulating vessel development in a patient having peripheral vascular disease, comprising delivering a delivery vector to the peripheral vascular system of the patient by intra-femoral artery injection directly into one or both femoral arteries, data very sumprising a transpone rading for a truncated VRF rubunit, and dapable of expressions the truncate. Were summit in the negligible mappales system, thereby promotion peripheral vascular development.

Preferably the delivery vector used in the invention is a viral delivery vector. In the preferred aspect, the delivery vector in the delivery vector in the delivery vector.

another preferred aspect, the delivery vector is an adenoassociated virus vector.

## Brief Description Of The Drawings

Figure 1 depicts the amino acid sequences of VEGF-B (SEQ I.D. NO. 1], VRF-2 [SEQ I.D. NO. 2], VEGF-C [SEQ I.D. NO. 3], FIGF :human PIGF-2: [SEQ I.D. NO. 4], VEGF-3 [SEQ I.D. NO. 5], poxylrus ORF-1 [SEQ I.D. NO. 6], and poxylrus ORF-2 [SEQ I.D. NO. 7]. Lower case letters signify signal peptides that are cleaved from the mature protein. The eight cysteines of the core sequence are underlined. Sequences are described in the following references: numan VEGF-B: Grimmond et al., Genome Res. 6:122-29 (1996); Clofsson et al., Proc. Nat'. Adad. Sci. U.S.A. 93:2567-81 (1996); mouse VEGF-B: Clofsson et al., Froc. 15 Natl. Acad. Sei. U.S.A. 93:2567-81 (1996); human VRF-2: Grimmond et al., Genome Res. 6:122-29 (1996); human VEGF-C: Joukov et al., EMBC J. 15:290-98 (1996); Lee et al., Proc. Natl. Adad. Sci. USA 93:1986-92 (1996); PlGF: Maglione et al., Oncogene 8:925-31 (1993); Hauser & Weich, Growth Factors 9:259-2.0 68 (1993); human VEGF3: PCT Application Serial No. PCT/US95/07283, published on December 12,, 1996, as WO95/39421; poxvirus ORF-1 and ORF-2: Lyttle et al., J. Virol. 68:84-92 (1994).

Figure 2a-2f depicts examples of truncated VRP amino acid sequences below the corresponding full length (F/L) VRP sequence. The amino acid sequences of each truncation are listed as follows:

2a(2) [SEQ I.D. NO. 19]; 2a(3) [SEQ I.D. NO. 20]; 2a(4)[SEQ I.S. NO. 21]; 2a(F/L) [SEQ I.D. NO. 37]; (1) [SEQ I.D. NO. 22}; 2d(2) [SEQ 1.D. NO. 23]; 2d(3) [SEQ 1.D. NO. 24]; 2d(4)[SEQ I.D. NO. 28]; 2e(F/L (SEQ I.D. NO. 38] (1) [SEQ I.D. NO. 26"; De(2) [SEQ I.D. No. 27]; 2e(3" [SEQ I.D. NO. 28]; 2e(4) (SEQ I.D. NO. 29); 2f(F/L) [SEQ I.D. NO. 39]; (1) [SEQ I.D. NO. 36); 2f(2) [SEQ I.D. No. 31]; 2f(3) [SEQ I.D. No. 32]; and Dr(4) [SEQ I.D. NO. 33].

#### Detailed Description Of The Invention 10 Construction of Novel Truncated VRP Sequences

In a first aspect the invention features a truncated VRP comprising at least one truncated VRP supunit. By "truncated VRP subunit" it is meant a VRP subunit having an amino acid 15 sequence substantially similar to one of the VRPs, for example, but not limited to, one of the sequences shown in Figure 1, or an analog or derivative thereof, wherein at least one of the Nterminal amino-acid residues N-terminal to the first cysteine of the core sequence of the mature subunit is deleted. A sequence that is "substantially similar" to a VRP comprises an amino acid sequence that is at least 25% homologous to the 8cysteine care sequence of VEGF-B, comprises all of the essential conserved cysteine residues of said core sequence, and retains VRP activity. By "truncated VRP subunit" is also meant a VRP subunit wherein at least one of the N-terminal amind add residues N-terminal to the first dysteine of the VEGF core sequence is deleted, and, at least one of the mystotnes in the miss sequence is deleted, wherein said monaths in a reasonnthi. A moneuropitall mysteine is the thin is not exquery, to menail VEL agricity. Assn. nonessential bysteines have been described in domnection with PDGF. (Potgens, et al. <u>J. Biol. Chem. 269:32879-85 (1994)</u>).

By "identity" is meant a property of sequences that what upon their limitarity or relationship. Identity is

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measured by dividing the number of identical residues by the total number of residues and multiplying the product by 100. Thus, two popies of exactly the same sequence have 100% identity, but sequences that are less highly conserved and have deletions, additions, or replacements may have a lower degree of identity. In calculating sequence identity, the two sequences are compared starting at the carboxy terminus of the N-terminal deletion. Those skilled in the art will recognize that several computer programs are available for determining sequence identity.

Analogs of a truncated VRP polypeptide or subunit are functional equivalents having similar amino acid sequence and retaining, to some extent, one or more activities of the related truncated VRP polypeptide or subunit. By "functional equivalent" is meant the analog has an activity that can be substituted for one or more activities of a particular truncated VRP polypeptide or subunit. Preferred functional equivalents retain all of the activities of a particular truncated VRP polypeptide or subunit, however, the functional equivalent may have an activity that, when measured quantitatively, is stronger or weaker, as measured in VRP functional assays, for example, such as those disclosed herein. In most cases, such truncated VRP polypeptides or subunits must be incorporated into a truncated VRP dimer in order to measure functional activity. Freferred functional equivalents have activities that are within 1% to 10,000% of the activity of the related truncated VRF polypeptide or subunit, more preferably setween 111 to 1000, and more preferrally within 50% to 2000.

The additing of a fortunative to ratain some distivity can be seem unreaded assistant techniques described herein. Consecutives include modification occurring during or after translation, for example, by phosphorylation, glycosylation, crosslinking, trylation, prescription pleature, linkage to an armorphy

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molecule, membrane molecule or other ligand (see Ferguson et al., 1988, Annu. Rev. Biochem. 57:285-320).

Specific types of derivatives or analogs also include amino acid alterations such as deletions, substitutions, additions, and amino acid modifications. A "deletion" refers to the absence of one or more amino acid residue(s) in the related polypeptide. An "addition" refers to the presence of one or more amino acid residue(s) in the related polypeptide. Additions and deletions to a polypeptide may be at the amino terminus, the carboxy terminus, and/or internal. Amino acid "modification" refers to the alteration of a naturally occurring amino acid to produce a non-naturally occurring amino acia. A "substitution" refers to the replacement of one or more amino acid residue(s) by another amino acid residue(s) in Derivatives can contain different the polypeptide. combinations of alterations including more than one alteration and different types of alterations.

While the effect of an amino acid change on VRP activity varies depending upon factors such as phosphorylation, glycosylation, intra-chain linkages, tertiary structure, and the role of the amino acid in the autive site or a possible allosteric site, it is generally preferred that the substituted amino acid is from the same group as the amino acid being replaced. To some extent the following groups contain amino acids which are interchangeable: the basic amino acids lysine, arginine, and histidine; the acidic amino acids aspartic and diutamib acids: the noutral colar amino acids serine, threinine, cysteine, flutamine, asparagine and, to a lesser extent, mothithing; the nonpolar aliphatic amine addis plyting, conting, valing, isolitating, and leating (nuwever, because of sine, divoine and alanine are more closely related and valine. isoffeuding and leuding are more closely related); and the or material amount and by cheay labanine, tryptophan, and tyrobine. ்ட நாழ்ந்துந்த இருக்கும் திறுத்தின் தெரியாக கொண்ணும் இந்துகுகுறியும்.

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alamine, glycine, and serine seem to be interchangeable to some extent, and cysteine additionally fits into this group, or may be classified with the polar neutral amino acids.

Preferred derivatives have one or more amino acid alteration(s) which do not significantly affect the activity of the related truncated VRP polypeptide or subunit. In regions of the truncated VRP polypeptide or subunit not necessary for VRP activity, amino acids may be deleted, added or substituted with less risk of affecting activity. In regions required for VRP activity, amino acid alterations are less preferred as there is a greater risk of affecting VRP activity. Such alterations should be conservative alterations. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent.

Conserved regions tend to be more important for protein activity than non-conserved regions. Standard procedures can be used to determine the conserved and non-conserved regions important for VRP activity using in vitro mutagenesis techniques or deletion analyses and measuring VRP activity as described by the present disclosure.

Derivatives can be produced using standard chemical techniques and recombinant nucleic acid molecule techniques. Modifications to a specific polypeptide may be deliberate, as through site-directed mutagenesis and amino acid substitution during solid-phase synthesis, or may be accidental such as through mutations in noste which produce the polypeptide. I hypeptides including derivatives can be obtained using changary techniques from as those described in Samprock et al., Milecular I wint, a li Speing Harbin Julicularly Press 1989. For example, Chapter 1: % demorpook describes procedures for side-durected mutagenesis of sloned CNA.

By a "roundated VPP prolymentate" is meant a polymentide tumblisting the animal transfer that are sense in a roundated VPP propunit

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of the invention, or a functional analog or derivative thereof as described herein. The term "truncated VRF polypeptide" also includes a truncated VRP supunit; the term subunit generally referring to a peptide that has been folded into an active three-dimensional structure.

By "truncated VEF" is meant a dimer of two VRP subunits. The two subunits may be derived from two different VRPs where both subunits are truncated VRP subunits. One or both of the subunits may be truncated; the two subunits may also have 10 different N-terminal deletions.

It is advantageous that the truncated VRP, truncated VRP subunit, or truncated VRF polypeptide be enriched or purified. By the use of the term "enriched" in this context is meant that the specific amino acid sequence constitutes a significantly 15 higher fraction (2 - 5 fold) of the total of amino acid sequences present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other amino acid sequences present, or by a preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term "significant" here is used to indicate that the level of increase is useful to the person making such an increase, and year-raily means an increase relative in other amino Acid legiendes it andio an least il bild, mole pracerbly at least " to I fill it gran mark. The term also been not imply that there is no amino acid sequence from other sources. The other source amino acid sequence may, for example, comprise amino upil minded by a yeart or Bacterial Menomo, or a ploning vestor John is nCCL. The next is next to sover him three

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situations in which man has intervened to elevate the proportion of the desired amino acid sequence.

It is also advantageous for some purposes that an amino acid sequence be in purified form. The term "purified" in reference to a polypeptide does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 10 fold greater, e.g., in terms of mg/ml). Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

In another aspect the invention features a nucleic acid molecule encoding a truncated VRP polypeptide or subunit.

In some situations it is desirable for such nucleic acid molecule to be enriched or purified. By the use of the term "enriched" in reference to nucleic acid molecule is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction (2 - 5 fold) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that ehriched does not imply that there are no other DNA or RNA Respendes present, hum that the relative amount of the whether the of idearest has real distributionally increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and renevally means on increase relative to other nucleic words of acount of Leigh D told, morn protorsolv at least 5 to 10 told at 1.0

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even more. The term also does not imply that there is no DNA or RNA from other sources. The other source DNA may, for example, comprise ENA from a yeast or bacterial genome, or a cloning vector such as pUC19. This term distinguishes from naturally occurring events, such as viral infection, or tumor type growths, in which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant to cover only those situations in which a person has intervened to elevate the proportion of the desired nucleic acid.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid molecule does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, e.g., in terms of mg/ml).

The nucleic acid molecule may be constructed from an existing VRP nucleotide sequence by modification using, for example, oligonucleotide site-directed mutagenesis, or by deleting sequences using restriction enzymes, or as described herein. Standard recombinant techniques for mutagenesis such as in vitro site-directed mutagenesis (Hutchinson et al., <u>J. Biol. Chem. 253</u>:6551, (1378), Sambrook et al., Chapter 15, supra), use of TAB® linkers (Pharmacia), and PCR-directed mutagenesis can be used to proste such mutations. The nucleic Acid mutagenesis can be used to proste such mutations. The nucleic Acid mutagenesis can be used to proste such mutations. The nucleic Acid mutagenesis can be used to proste such mutations.

The invention als relatives combinant DNA vestrs and recombinant DNA expression vestors preferably in a sell or an organism. The recombinant DNA vestors may contain a sequence tribulation a truncated UCC or a constraint derivative thereof the contain assertion of the contains.

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transcription in a nost cell. The recombinant DNA vector can contain a transcriptional initiation region functional in a cell and a transcriptional termination region functional in a cell.

The present invention also relates to a cell or organism that contains the above-described nucleic acid molecule or recombinant DNA vector and thereby is capable of expressing a truncated VRP peptide. The polypeptide may be purified from cells which have been altered to express the polypeptide. A cell is said to be "altered to express a desired polypeptide" when the cell, through genetic manipulation, is made to produce a protein which it normally does not produce or which the cell normally produces at lower levels. One skilled in the art can readily adapt procedures for introducing and expressing either 15 genomic, cDNA, or synthetic sequences into either eukaryotic or prokaryotic cells.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. precise nature of the regulatory regions needed for gene sequence expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis thitiation. Just regions will normally include those 5'-nona ting degrences invitaria with initiation in transcription and translation, such is the TATA box, targing componen, JAAT sequence, and the like.

For example, the entire coding sequence of a truncated VRP subjunct or a fragment therein, may be sumbined with the is more of the following in an appropriate expression result of allow

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for such expression: (1) an exogenous promoter sequence (2) a riposome binding site (3) a polyadenylation signal (4) a secretion signal. Modifications can be made in the 5'untranslated and 3'-untranslated sequences to improve 5 expression in a prokaryotic or eukaryotic dell; or codons may be modified such that while they encode an identical amino acid, that codon may be a preferred codon in the chosen expression system. The use of such preferred codons is described in, for example, Grantham et al., Nuc. Acids Res., 9:43-74 (1981), and Lathe, J. Mol. Biol., 183:1-12 (1985) hereby incorporated by reference herein in their entirety.

If desired, the non-coding region 3' to the genomic VRP sequence may be operably linked to the nucleic acid molecule encoding such VRP subunit. This region may be used in the recombinant DNA vector for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the ENA sequence encoding a VRP gene, the transcriptional termination signals may be provided. Alternatively, a 3' region functional in the host cell may be substituted.

An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene sequence expression. Two DNA sequences (such as a promoter region sequence and a truncated VRP sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not I result in the introduction of a frame-shift mutation in the ording sequence, (1 interfere with the ability of the promoter strion requence to direct the transcription of a trincated VAR gains sequence, or (3) interfere with the ability of the a truncated VRP gone sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linear to a PNM sequence if the primoter were damable of entértica tesperaintien eficati CDA réquence. Thus, to surrocc

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a truncated VEP gene, transcriptional and translational signals recognized by an appropriate host are necessary.

## Expression and Purification of Novel Truncated VRP Sequences

Examples I and 3 describe the expression and purification of novel truncated VRP sequences of the present invention as expressed in baculovirus systems. Those skilled in the art will recognize that the truncated VRPs of the present invention may also be expressed in other cell systems, both prokaryotic and eukaryotic, all of which are within the scope of the present invention. Examples 4+6 provide examples of suitable assays for functional activity of the novel truncated VRPs.

Although the truncated VFPs of the present invention may be expressed in prokaryotic cells, which are generally very efficient and convenient for the production of recombinant proteins, the truncated VRPs produced by such cells will not be glycosylated and therefore may have a shorter half-life in vivo. Prokaryotes most frequently are represented by various strains of E. coli. However, other microbial strains may also be used, including other bacterial strains. Recognized prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, and the like. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

In prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host may be used. Examples of suitable plasmic vectors may include pBRSSI, pUCLIS, pUCLIS and the like; ...ttable phase to bacteriophage vectors may include yqtl0, yqtl1 and the like; and suitable virus vectors may include pMANner, pRES and the like. Preferably, the selected vector of the present invention has the capabity to replicate in the selected noot bell.

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necessary to operably link the truncated VRP sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible. Examples of constitutive promoters include the interconoter of bacterisphage  $-\lambda$ , the bla promoter of the  $\beta$ -labtamase dene sequence of pBR321, and the CAT promoter of the chloramphenical acetyl transferase gene sequence of tPR325, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage  $\lambda$ (Fi and Fe), the trp, recA,  $\lambda$ acl,  $\lambda$ acl, and gal promoters of E. ccli, the  $\alpha$ -amylase (Ulmanen et al., J. Bacteriol. 162:176-132(1985). and the q-23-specific promoters of B. subtilis (Gilman et at., Gene sequence 30:11-20(1984)), the promoters of the bacteriophages of Bacillus (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)), and Streptomyces promoters (Ward et at., Mcl. Gen. Genet. 203:468-479(1986)). Prokaryotic promoters are reviewed by Glick (J. Ind. Microbiot. 1:277-282(1987); Cenatiempo (Biochimie 68:505-516(1986)); and Gottesman (Ann. Rev. Genet. 18:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene sequence-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold et at. (Ann. Rev. Micropiol. 35:365-404(1981)). The ribosome binding site and other sequences required for translation initiation are operably linked to the nucleit acid molecule coding for the truncated TRIC by, for swample, in frame lightin to synthetic lightin to synthetic lightine in prokaryot: soils, no signal peptide sequence is required. The selection of control sequences, expression vectors, transformatics methods, and the like, are dependent on the type of nost cell used to express the gene.

has ased herein, "obl!", "bell line", and "bell dulnume"

progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom, without regard to the number of transfers. Truncated VRF peptides expressed in prokaryotic cells are expected to comprise a mixture of properly truncated VRP peptides with the M-terminal sequence predicted from the sequence of the expression vector, and truncated VRP peptides which have an N-terminal methionine resulting from inefficient cleaving of the initiation methionine during bacterial expression. Both types of truncated VRP peptides are 10 considered to be within the scope of the present invention as the presence of an N-terminal methionine is not expected to affect biological activity. It is also understood that all progeny may not be precisely identical in DNA content, due to 13 deliberate or inadvertent mutations. However, as defined, mutant progeny have the same functionality as that of the originally transformed cell.

Preferred prokaryctic vectors include plasmids such as those capable of replication in E. coil (such as, for example, 20 pBR322, ColEl, pSC101, pACYC 184,  $\pi$ VX. Such plasmids are, for example, disclosed by Sambrook (of. "Molecular Cloning: A Laboratory Manual", second edition, edited by Sambrock, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)). Bacillus plasmids include pC194, pC221, pT127, and the like. 2.5 Such plasmids are disclosed by Gryczan (In: The Molecular Biology of the Bacilli, Academic Fress, NY (1982), pp. 307-329). Cuitabla Ctreptomydes plasmids inplude pl3101 (Wendall wh al., <u>I. Babremiol.</u> [e3:41] T-4183 [1987] , and otrontomyces distorishings such is  $\phi$ 231 (thater of al., In: dixih international dymposium on Actinomycetaies Biology, Akademiai Haido, Budapest, Hungary (1996), pp. 45-54). Pseudomonas plasmids are reviewed by John on al. (Rev. Infect. Dis. 9:693-754/1346 , and Image Copies [...Bioteriol. 23:724-74281/1465.

Eukaryotic host cells which may be used in the expression systems of the present invention are not strictly limited, provided that they are suitable for use in the expression of the truncated VSP peptide. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, mammalian cells either in vivo, or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO or CHO-KI, or cells of lymphoid origin and their derivatives.

The truncated VRPs of the present invention may also be expressed in human cells such as human embryo kidney 293EBNA cells which express Epstein-Barr virus nuclear antigen 1, as described, for example, in Olofssen, B. et al., Proc. Natl. Acad. Sci. USA 93:2576-2581 (1996). The cells are transfected with the expression vectors of Example 2 by using calcium phesphate precipitation, and the cells are then incubated for at least 48 hours. The truncated VRP peptides may then be purified from the supernatant as described in Example 3.

In addition, plant cells are also available as hosts, and control sequences compatible with plant cells are available, such as the cauliflower mosaic virus 35S and 19S, and nepaline synthase promoter and polyadenylation signal sequences. Another preferred host is an insect cell, for example the Drosophila larvae. Using insect cells as hosts, the Drosophila alcohol dehydrogenase promoter can be used. Rubin, Spience 246:1453-1459(1988).

Any of a series of years gene sequence expression systems can be distinged which interpreted promoter and termination elements from the actively expressed gene sequences adding for glycolytic encymes are produced in large quantities when years are grown in mediums sich in glucose. Known glycolytic gene sequences can also provide very efficient transcriptional element displayer. We so provides constantial advantages in that the can also provide acceptantial advantages in that

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A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene sequence products and secretes peptides bearing leader sequences (i.e., pre-peptides). For a mammalian host, several possible vector systems are available for the expression of truncated YRP peptides.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of 20 the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Expression of truncated VRPs in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the unitizin of PMA synthesis. Englorned aukanyotab promoters include, for example, the primiter of the molie metallichionoin I jame sequence (Hamer et al., <u>1. M.). Appl. Gen</u>. 1:273-Isek(1981); the TK primoter it Horpes virus (McKnight, Cell 31:355-365 (1982)); the 3740 early promoter (Benoist et al., Nature Linksh 1992 4-31200341 / the peach sall gene Mangages commess todance of the test the Ansa The

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TUSA: 79:6971-6975(1982); Silver et al., Proc. Natl. Acad. Sci. VDSA: 81:5951-5955 (1984)).

Translation of eukaryctic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryctic promoter and a DNA sequence which encodes a truncated VRP (or a functional derivative thereof does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as the truncated VRP coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the truncated VRP coding sequence).

A truncated VRP nucleic acid molecule and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced DNA sequence into the host chromosome.

A vector may be employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their shromosomes can be selected by a.s. introducing one or more markers which allow for beleation of hoot cells which contain the expression vector. The marker may provide for protectophy to an electrophy nost, little resistance, e.g., antibities, of nearly metals, such as copper, or the like. The selectable marker gene sequence can either be directly linked to the DNA sene sequences to be expressed, in introduced into the same cell by contransportion. Administrate elements may also be

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needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include 5 those described by Okayama, Molec. Cell. Biol. 3:280 (1983).

The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of 10 importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vactor; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art (Botstein 20 et al., Miami Whtr. Symp. 19:265-274(1982); Broach, In: "The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, Cell 23:203-204 (1982); Bollon et al., J. Clin. Hematol. Oncol. 10:39-48 (1980); Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-698(1980).

under the vester is nuclear asid molecule containing the Condition is not lived propaged but expression, the INA Construct a may be entropied into an age polate a so call by any of a variety of suitable means, i.e., transformation, transfection, conjugation, protoplast fusion, electroporation, particle quin teannology, lipofestion, calsium phosphate pracipitation, direct midrithration, DEAE-destrai

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transfection, and the like. The most effective method for transfection of eukaryotic cell lines with plasmid ENA varies with the given cell type. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene molecule(s) results in the production of truncated VRP or fragments thereof. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). A variety of incubation conditions can be used to form the peptide of the present invention. The most preferred conditions are those which mimic physiological conditions.

Production of the stable transfectants, may be accomplished by, for example, transfection of an appropriate cell line with an eukaryotic expression vector, such as pCEP4, in which the coding sequence for the truncated VRP polypeptide or subunit has been cloned into the multiple cloning site. These expression vectors contain a promoter region, such as the human cytomegalovirus promoter (CMV), that drive high-level transcription of desired DNA molecules in a variety of mammalian cells. In addition, these vectors contain genes for the selection of cells that stably express the DNA molecule of interest. The selectable marker in the pCEP4 vector encodes an enzyme that confers resistance to hygromycin, a metabolic inhibitor that is added to the culture to kill the nontransfected cells.

Cells than have stably interporated the transfected DNA will be identified by their resistance to acception model, as assembed above, and simple real lines will be produced by expansion of resistant solutions. The expression of the truncated VRPs DNA by those cell lines will be assessed by a latter hybridization and Northern plot analysis.

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# Pharmaceutical Compositions and Therapeutic Uses

One sojest of this invention is to provide trunsated VRP in a pharmaceutical composition suitable for therapeutic use. Thus, in one aspect the invention provides a method for stimulating angiogenesis in a patient by administering a therapeutically effective amount of pharmaceutical composition comprising a truncated VRP.

By "therapeutically effective amount" is meant an amount of a compound which produces the desired therapeutic effect in a patient. For example, in reference to a disease or disorder, it is the amount which reduces to some extent one or more symptoms of the disease or disorder, and returns to normal, either partially or completely, physiological or blockemical parameters associated or causative of the disease or disorder. 15. When used to therapeutically treat a patient it is an amount expected to be between 0.1 mg/kg to 100 mg/kg, preferably less than 50 mg/kg, more preferably less than 10 mg/kg, more preferably less than 1 mg/kg. The amount of compound depends on the age, size, and disease associated with the patient.

The optimal formulation and mode of administration of compounds of the present application to a patient depend on factors known in the art such as the particular disease or disorder, the desired effect, and the type of patient. While the compounds will typically be used to treat human patients, they may also be used to treat similar or identical diseases in other vertebrates such as other primates, farm animals such as swine, cattle and poultry, and sports animals and pets such as nerser, duco and bats.

firelegably, and the learning directive amount is provided as a pharmaceuticul torpolation. A praimacel i dul Adent or composition refers to an adent or composition in a form suitable for administrative into a multicellular organism such as a human. Suitable porms, in part, depend upon the use vá the plube il entrý, fil example, bal, transpermal, i by

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injection. Such forms should allow the agent or composition to reach a target cell whether the target cell is present in a multicellular host or in culture. For example, pharmacological agents or compositions injected into the blood stream should E se soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the agent or composition from exerting its effect.

The claimed compositions can also be formulated as pnarmaceutically acceptable salts (e.g., acid addition salts) and/or complexes thereof. Pharmaceutically acceptable salts are non-toxic salts at the concentration at which they are administered. The preparation of such salts can facilitate the pharmacological use by altering the physical-chemical characteristics of the composition without preventing the composition from exerting its physiological effect. Examples of useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate the administration of higher concentrations of the drug.

Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, prospnate, sulfonate, sulfamate, sulfate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclolexylsulfonate, 25 cyclohexylsulfamate and quinate. Pharmaceutically acceptable salts can be obtained from acids such as hydrochloric acid, sulfurio abid, phospheric adid, sulfonio abid, sulfamid adid, abetic acia, ditric acia, lactic sold, tartario apid, malonio with, methem-buli-nit acts, ethanesulfinis r=tulidenestijpurio symbole median acid, cyclobexylsulfamic acid, and quinic abid. Such salts may be prepared by, for example, reacting the free abid in pase forms of the product with one or more App. Valente (d. ne approprioto oggo or abid in a bolvent or

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medium in which the salt is insoluble, or in a solvent such as water which is then removed in vacuo or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin.

Carriers or excipients can also be used to facilitate administration of the compound. Examples of carriers and excipients include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or suchose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. The compositions or pharmaceutical composition can be administered by different noutes including intravenously, intraperatoreal, subcutaneous, and intramuscular, orally, topically, or transmucosally.

The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitel and sorbitel), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

The compounds of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co., Easton, PA, 1990. See also Wang, Y.J. and Hanson, M.A. "Parenteral Formulations of Froteins and Peptides: Stability and Stability and Stability and Stability and Stability and Stability and Stability." Journal of Parenteral Science and Technology, Februical Februar No. 1., Josep. 42:22 (1984). A statusle samunication of corman may asked a determined by a measual prestitioner for each patient individually.

For eystemic administration, innection is preferred, e.g., intramuscular, intravenous, intraversus, fittendeal, subcutaneous, intraversus, respectively. Fit injection, the

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compounds of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. Alternatively, the compounds of the invention are formulated in one or more excipients (e.g., propylene glycol) that are generally accepted as safe as defined by USP standards. They can, for example, be suspended in an inert cil, suitably a vegetable oil such as sesame, peanut, plive oil, or other acceptable carrier. Preferably, they are suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 5.6 to 7.4. These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH buffering agents. Useful buffers include for example, sodium 15 acetate/acetic acid buffers. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the molecules can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the identiation. Such percerants are decerally known in the art, and include, for example, for transmissail administration, base maits and fulfill activenives. In addition, denorgents may be need to facilitate becomeation. Tianumucosal administration may be, for example, through hasal sprays or ising suppositories. For oral administration, the molecules are cormulated inc. Placentional oral administration locage intry hade as sabsules, reclets, and limite preparations

For topical administration, the compounds of the invention are formulated into bintments, salves, gels, or creams, as is generally known in the art.

If desired, solutions of the above compositions may be thickened with a thickening agent such as methyl cellulose. They may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acadia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether alconol sulfates or sulfanates, e.g., a Triton).

Compositions useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be simply mixed in a blender or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

The amounts of various compounds of this invention to be administered can be determined by standard procedures. Senerally, a therapeutically effective amount is between about 1 nmole and 3 μmole of the molecule, preferably between about 10 nmole and 1 μmole depending on the age and size of the patient, and the disease or disorder associated with the patient. Senerally, it is an amount between about 0.1 and 50 mg/ks, preferably 1 and 20 mg/ks of the animal to be treated.

For use by the physician, the compositions will be provided in disage unit form clutaining an amount or a tributate. The, VRE polypepties, or VRE accumit.

#### Gene Therapy

A truniaged VFF or incompanies organized with also be well above the second of the second constant  $x_i$ 

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(1992). Miller states that advances have resulted in practical approaches to human gene therapy that have demonstrated positive initial results. The basic science of gene therapy is described in Mulligan, Science 260:926-931 (1993). One example of dene therapy is presented in Example 7, which describes the use of adenovirus-mediated gene therapy.

As another example, an expression vector containing the truncated VRP ocding sequence may be inserted into cells, the calls are grown in vitro and then injected or infused in large 10 numbers into patients. In another example, a DNA segment containing a promoter of choice (for example a strong promoter) is transferred into cells containing an endogenous truncated VRP in such a manner that the promoter segment enhances expression of the endogenous truncated VRP gene (for example, the promoter segment is transferred to the cell such that it becomes directly linked to the endogenous truncated VRP gene).

The gene therapy may involve the use of an adenovirus vector including a nucleotide sequence coding for a truncated VRP subunit, or a naked nucleic acid molecule coding for a truncated VRP subunit. Alternatively, engineered cells containing a nucleic acid molecule coding for a truncated VRP subunit may be injected. Example 7 illustrates a method of gene therapy using an adenovirus vector to provide angiogenesis therapy.

Expression vectors derived from viruses retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA virises, or bovine papilloma virus, may be used for delivery of nucleotide sequences (e.g., SPNA: embeding resemblinant orundated VRS subunit into the targeted deli population. Methods which are well known to those skilled in the art can be used to construct recombinant wiral vectors containing coding sequences. See, for example, the termiques described in Maniatis et al., Molegular Cleming: A Lastinatory Manual, John April: Marcor Makeratory, N.Y.

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(1989), and in Ausubel et al., <u>Current Protocols in Molecular Biology</u>, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system <u>e.g.</u>, liposomes or other lipid systems for delivery to target cells (<u>See e.g.</u>, Felgner et al., Nature 337:387-8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins. <u>See</u>, Miller, Nature 357:458-60, 1992.

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the recleus of a cell, through a process of microinjection. Capecchi MR, Cell 22:479-88 (1980). Once recombinant genes are introduced into a cell, they can be recognized by the cells normal mechanisms for transcription and translation, and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with calcium phosphate and taken into cells by pinocytosis (Chen C. and Okayama H, Mol. Cell Biol. 7:2745-52 (1987)); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu G. et al., Nucleic Acids Res., 15:1311-26 (1987)); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell 'Felgher PL., et al.. Proc. Natl. Arad. Doi: D&A. - s4:T413-7 - 1947. ; And Cartiuse compardment quint INA bland to small the education. Tand NC. et al., Proc. Natl. Acad. Scu. +7:3168-71 (1993). Another method fds introducing DNA into ceils is to couple the DNA to chemically modified proteins.

It has also been whown that adentwirus proteins are dapacle of destabilizin: education and enhancing the uplace of

DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the 5 recombinant gene. Curiel DT et al., Am. J. Respir. Cell. Mol. Biol., 6:247-52 (1992).

In addition, it has been shown that adeno-associated virus vectors may be used for gene delivery into vascular cells Gnatenko, D., J. of Invest. Med. 45:87-97, (1997)).

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or 15 enzymatically active RNA. Sene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid molecule contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid molecule into the 20 cell through the membrane or by endocytosis, and release of nucleic acid molecule into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid molecule into the nucleus of the cell and binding to appropriate nuclear factors for 25 transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the refinition of gene transfer as used nergin and appointfully betark to bene transfer to express a therapeutic product from a sell in vivo is in vitre. Gene tindisfer can be parformed as vivo in balls which as sinch transplanted into a patient, or can be performed by direct administration of the nucleic acid molecule or nucleic acidprotein complex into the patient.

In another preferred empodiment, a vector having nucleic acid molecule sequences encoding a truncated VRP is provided in which the nucleic acid molecule sequence is expressed only in a specific tissue. Methods of achieving tissue-specific gene expression as set forth in International Publication No. WO 93/09236, filed November 3, 1991 and published May 13, 1993.

In another preferred embodiment, a method of gene replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid molecule sequence which is capable of being expressed in vivo in an animal and thereby providing or augmenting the function of an endogenous gene which is missing or defective in the animal.

In all of the preceding vectors set forth above, a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

#### Examples

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To assist in understanding the present invention, the following Examples are included which describes the results of a series of experiments. The experiments relating to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the corps of the invention as described horses and hereinafter limed.

#### Example 1

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Cloning of N-Terminally Truncated VEGF-5, (des-(1-20)-b21-VEGF-8 (or des(2-21)-VEGF-B).

In order to create a novel VEGF-B-related protein that lacks the first 20 amino acids, a cDNA construct is created in the following manner:

A SNA encoding human VEGF-B is amplified from a human heart or skeletal muscle cDNA), or a human fetal brain cDNA library, or a cDNA preparation from another suitable human tissue source by FCR with bligonucleotides corresponding to the published sequence of human VEGF-E. Using standard molecular biology techniques (Sambrock et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor NY), a DNA fragment then is generated that encodes at its 5' end the signal sequence of human VEGF-B, followed by a codon for proline, the first amino acid residue in mature VEGF-B, and then followed by codons corresponding to amino acids from residues 32 to the C-terminus of human VESE-B, followed by a stop codon. Appropriate additional non-coding nuclectide sequences are added to the 5' and 3' ends of this DNA construct so as to allow insertion of the DNA into an appropriate expression vector.

In this manner the cleavage site for the signal peptide is preserved in a manner identical to that found in native VESF-B. However, this strategy results in a change in the new N-terminal amino acid of the truncated VEGF-B. Whereas the normal D-terminal amin. Acid maxidue in des 1-21:-VFSF-B is a typicine recline:

magnificallylarings[SVa]FNJapH1ERVJFWIJVI $\underline{\mathbf{x}}$ TPAT, the new N-terminal amine the Lagranian, and the regulting truncated VEGF-B is equivalent to des(2-21-VEGF-B):

mobilirrlllvallclartcaPTRAT...

The mange from the native amin' while if the truncated  $35.35^{-19}$  throughout in the Nable of a  $37.7^{-19}$  throughout from

not expected to have any effect on the biological activity of the truncated VEGF-B. The advantage of this strategy is that the signal peptide sequence is maintained thus ensuring efficient cleavage of the signal peptide from the precursor during protein processing/secretion.

In another example, truncated VEGF-B, des(1-13)-VEGF-B, is constructed by deleting the first 15 amino acids. The signal peptide cleavage site would be preserved in this case because residue#15 and residue#1 (the new and old N-termini) are identical (proline):

mspllrrillvallclartqa[PV3QFDGPSHQKKVV]PWIDVYTRAT...

msplirrillvallqlartqaPWIDVYTRAT...

One of skill in the art would understand that other signal peptides may be used in the present invention. For example, the signal peptide of VEGF-B or VEGF-C could be used which would require that the first amino acid of the truncated protein be an alanine or glycine, respectively, in order to preserve the respective signal peptide cleavage sites. A further alternative would be to use signal peptide sequences from other known proteins; some of these may have cleavage sites compatible with the N-terminal tyrosine of the truncated des(1-20)-VEGF-B.

Another alternative would be to generate a construct that encodes a precursor protein with a cleavage site that incorporates two, rather than one, amino acids from the N-terminus of the original VESF-B protein sequence. The purpose of this strategy would be to ensure more fully that the cleavage size is asympatible with sizes peptiase function.

1. This would increases two new amino trees at the S-terminus of the truncated VEGF-B sequence but such a change would not be expected to alter prological function of the truncated portice.

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The strategy described to generate DNA for expression of desci-20)-VEGF-B is useful for generation in an analogous manner of VEGF-B mutants with N-terminal truncations of other desired lengths. Further, the strategy is useful to generate N-terminal truncations of other desired lengths in other VEGF-related forms and their isoforms of other species.

## Example I: Expression Of N-Terminally Truncated VEGF-B Subunits

The DNA fragment encoding truncated VEGF-B from Example 1 may be cloned into a suitable plasmid vector.

Sf9 (Sporoptera frugiperda) cells are co-transfected with baculovirus transfer vector pAcUW51 containing cDNA encoding truncated VESF-B and baculovirus (Baculogold, Pharmingen, San Diego, CA). Selection and plaque purification of recombinant virus are performed according to established protocols using Blue agar everlays (Gibco BRL). High stock of recombinant virus is produced in exponentially growing Sf9 cells using a multiplicity of infection of 0.05. For expression of truncated VESF-B, Sf9 cells (1x106 cells/ml) growing in serum free medium are infected with recombinant virus at a multiplicity of 10. Supernatant is collected after 72 hours post infection. VEGF expression in baculovirus-infected insect cells, which can be used to express the truncated VRPs of the present invention is also described in Fiebich et al., (Eur. J. Biochem. 211: 19-26, 1993). In this system, VEGF has been shown to be produced in high yield, with efficient glycosylation similar to that seen in mammalian cells. In fact, those skilled in the art will recognize than expression in other systems, including mammalian well expression systems, is considered to do within the scope ut this invention. Methods of expressing VEGF pritisins which can be used to express the truncated VRPs of the present invention using baculovirus systems are also provided in throughness which describe VEGF expression, for example, 4.3 

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(Baculovirus Expression Vectors: A Laboratory Manual [W.H. Freeman, New York!, 19924.

Those skilled in the art will recognize that other expression systems may also be used to express functionally active truncated VRPs.

Functionally active recombinant VEGF isoforms have been expressed in E. Coli (Wilting et al., Dev. Biol. 176, 76-85, 1996; from inclusion body by refolding according to the pricedure described previously for homo- and heterodimers of FDGF (Schneppe et al., Gene 143, 201-09, 1994) and in yeast (Mchanraj et al., Biochem. Biophys. Res. Commun. 215:750-56, 1995).

Still other methods of expressing VEGF which can be used to express VRPs in the present invention are described, for example, in Jasny, Science 238:1653, 1987; and Miller et al., In: Genetic Engineering, 1986), Setlow, J.K., et al., eds., Plenum, Vol. 8, pp. 277-297).

## Example 3: Purification Of Recombinant Truncated VRPS

For purification of the baculovirus-expressed truncated VEGF-B of Example 2 from insect-cell supernatant, a number of standard techniques can be used. These techniques include, but are not limited to ammonium sulfate precipitation, acetone precipitation, icn-exchange chromatography, size exclusion 25 chromatography, hydrophobic interaction chromatography, reverse-phase HPLC, concanavalin A affinity chromatography, in electric fromsing, and chromatoficusing. Other standard protein guidication termiques are readily obvious to one Stalled in the Art. - You example, proteins with specific hads, which is nictimine days, altoyen days, etc., scala be produced by engineering DNA ancoming such tags into the VEGF-8 DNA such that proteins containing said tags in a manner compatible with the protein's prelociall activity would be expressed and కా,సార్వాణులో త్రారం ఇ<del>క్కి అన్కారు. గుండు అం</del>దరు మూ<mark>డు ఉన్నా చేశ్వా మన్నాడన్నారు. కార్స్ కానుడు. . కార్స్</mark>న్స్

methods are considered within the scope of the present invention.

A preferred purification method for truncated forms of VESF-B is described in the following: Sf9 Cell supernatant is centrifuged at 10000 rpm for 30 minutes to remove cell debris and viral particles. Supernatant is then concentrated and dialyzed against 20 mM Tris (pH 8.3) for 24 hours. The dialyzed supernatant is centrifuged again to remove insoluble material and loaded onto a Sepharose Q anion exchange column. 10 Protein is eluted from the column by gradient elution using a gradient of NaCl (0 - 1 M NaCl). Chromatography fractions are analyzed by SDS polyadrylamide gel electrophoresis and by ELISA using an antibody that recognizes VEGF-B. Fractions with VEGF-E immunoreactivity are pooled, concentrated, and dialyzed 15 overnight against 0.1% trifluoroacetic acid. Material so prepared is further purified by reverse phase HPLC. Typically approximately 2-5 mg of protein is loaded on a semipreparative C4 column and eluted with a gradient of acetonitrile in 0.1% trifluoracetic acid as described in Esch et al., Meth. Enzymol. 103, 72-89, 1983. Fractions containing truncated VEGF-B are 20 pooled and stored at -80 degrees Celsius until further use.

A preferred method of purification of the basic and heparin-binding N-terminally truncated forms of VESF-related protein subunits and analogs thereof includes the combined use of heparin-Sepharose affinity chromatography and cation-exchange chromatography, optionally followed by reverse-phase HPLT, essentially as nesocribed in Connolly of al., <u>C. Biol. Dhen. Audicioli7-14</u>, 1944, Gospodarowicz et al., (<u>Proc. Natl. Adad. Jol. USA</u>, 46:7311-11, 1969, or flower et al., <u>Embo J. Embo J.</u>

Furthidation is monitored by following the elution of VRF-like material using a number of techniques including constrate proper assay while  $\frac{128}{3}$  Flateled VRF and receptor

preparations consisting of cells or cell membrane preparations in functional assays as described in Examples 4-6.

The truncated VRPs expressed in other eukaryotic cell systems such as yeast or mammalian cells, may be purified in the same manner.

Truncated VRPs expressed in proxaryotic cells will likely need to undergo a re-folding step for proper dimerization of subunits, as described in, for example, Schneppe et al., (Gene 143:201-09, 1994).

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#### Example 4: Receptor-Binding Assay

The binding of truncated VRFs to VEGF receptors can be assessed in various ways. Useful methods include the determination of the ability of VRP analogs to bind to endothelial cells or to cells artificially transfected with KDR, or to soluble forms of the KDR receptor (for example, a KDR/alkaline phosphatase fusion protein (Gitay-Goren et al., <u>J. Biol. Chem.</u> 271:5519-23 (1996)). A preferred procedure has been described by Terman et al. (<u>Biochem. Biophys. Res. Commun.</u> 137:1573-86, 1992).

In this procedure, KDR cDNA is transferted into CMT-3 monkey kinney cells by the DEAE-dextran method by incubating plated cells with DMEM containing 1  $\mu g/ml$  DNA, 0.5  $\mu g/ml$  DEAE dextran, and 100  $\mu$ M chloroquine. Following incubation for 4 hours at 37 degrees Celsius, the medium is aspirated and cells are exposed to 10% DMSO in FES for one minute. The cells are then washed white with IMEM containing 10% call serum and then which short if 9.4  $\pm$  mark at 27 degrees delai a in IMEM/100 call person containing 11%  $\mu$ M CACLES

30 VEGF-B is radificated daing either the lodogen method or the chloramine T method. Radiolahelled VEGF-B to reparated from axcess tree todine-125 using gel filtration on a Separader 425 moltan or a negation-department author. Opening activity of

order of 10° ppm/ng. For radioceptor assays, CMT-3 (10° cells/well) are plated in 12-well plates. Twenty four hours later, cells are washed twice with PBS, and 0.5 ml of DMEM containing 0.15° gelatin and 25 mM HEPES, pH 7.4 is acced. 1051-VEGF-B, at concentrations ranging from 1-500 pM, is then added. Binding experiments are done in the presence or absence of 0.3 nM unlabeled VEGF-B for the determination of specific binding. After a 90-minute incubation at room temperature, a 50 μl sample of the media from each well is used to determine the concentration of free radioligand, and the wells are washed 3 times with ice cold PBS containing 0.1% BSA. Cells are extracted from the wells by incubation for 30 minutes with 1% Triton X100 in 100 mM sodium phosphate, pH 3.0, and the radioactivity of the extract is determined in a gamma counter.

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# Example 5: Mitogenic Assay

The mitogenic activity of truncated VRPs on endothelial cells of human or mammalian origin can be determined by a number of different procedures, including assays where cell proliferation is measured by growth of cell numbers or by incorporation of radioactive DNA precursors (thymidine incorporation) or otherwise appropriately labeled DNA precursors (brome-decayuriding incorporation). These and other methods generally used to determine cell proliferation, including those methods where mitogenic activity is assessed in vivo (for example by determining the mitotix index of emacthelial cells) are considered within the scope of this invention. A preferred method is described herein (Bahlad of al., <u>Fro: Natl. Adad. Dol. FUA</u> 41:5344-66, 1994 : Hivino arxtic arch engothelial delig maintained in stock dultured in the rresence of Dulbacer's modified Eagle's medium supplemented with 100 calf sorum and antibioning transpoin at 30 µg/ml and i milaine at lilb µguml, and ructi fiproblast drowth factor (1-

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ratio of 1:64. For mitigenic assays, cell monolayers from stock plates (at passages 3-12) are dissociated using trypsin. Colls are then seeded at a density of approximately 3000 cells/well in 24-well plates in the presence of DMEM and antibiotics as described above. Samples to be assayed (1-10  $\mu\text{l})$ , appropriately diluted in DMEM/0.1% boving serum albumin), are added six nours after plating of cells and again after 4% hours. After 4 days of culture, endothelial cells are detached from plates with trypsin and counted using a Coulter particle counter.

Another mitogenic activity assay is provided in Clofsson, B. et al., Frcc. Natl. Acad. Sci. USA 93:2576-81, 1996). Second passage human umbilical vein endothelial cells (HUVECs) are plated into 96-well plates (4 X  $10^3$  cells per well) in M-199 medium supplemented with  $10^3$  (vol/vol) fetal bovine serum and incubated for 24 hours. Cell culture conditioned medium containing the truncated VRP, in the presence of 1-10  $\mu$ g/ml heparin, or purified truncated VRP is added to the HUVECs, and the cells are stimulated for 43 hours. Fresh cell culture conditioned medium containing [ $^3$ H] thymidine (Amersham; 10  $\mu$ Ci/ml) is added to the cells and stimulation is continued for another 48 hours. Cells are washed with PBS and trypsinized and the incorporated radioactivity is determined by liquid scintillation counting. The activity of truncated VRP is compared to the activity of non-truncated VRP.

In another alternative method, bovine capillary endothelial SCE) bells are seeded into 04-well plates and forwh until confluence in minimal essential medium (MEM) supplemented with 10° fetal balf serum. Cells are starved in MEM supplemented with 3° fetal balf serum for 70 hours, after which conditioned medium diluted into cerum-free medium is added to the bells and the bells are stimulated for 04 hours. The objects in includes course the last 4 hours.

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with NaOH, and incorporated radioactivity is determined by liquid scintillation counting. The activity of truncated VRP is compared to that of non-truncated VRP. Bovine fibroblast growth factor (p-FGF) may be used as an additional control for 5 mitogenic activity, and may also be used to measure its potentiating activity of truncated VRP activity.

## Example 6: Angiogenic Activity Of Truncated VRPS

The angiogenic activity of substances can be determined 10 using a variety of in vivo methods. Commonly used methods include the chick choricallantoic membrane assay, the corneal bouch assay in rabbits, rats, or mice, the matrigel implant assay in mice, the rabbit ear chamber andiogenesis assay, the hamster cheek pouch assay, the Hunt-Schilling chamber model and 15 the rat sponge implant model. Other assay methods to assess the formation of new blood vessels have been described in the literature and are considered to be within the scope of this invention.

A preferred method for demonstrating the angiogenic activity of truncated VEPs is the rabbit corneal pouch assay. 20 In this assay, Elvak (ethylene vinyl acetate) polymer pellets containing approximately 1-1000 ng of the growth factor and a constant amount of rabbit serum albumin as carrier is implanted into a surgical incision in the cornea as described in more detail in Phillips and Knighton, Wound Rep. Reg. 3, 533-539, 1995; Gimbrone et al., J. Natl. Canc. Inst. 52:413-27, 1974; Risau, Proc. Natl. Acad. Sci. USA 83:3855-52, 1986'. Growth rattor-inqueed vascularination of the cornea is then observed over a period of D weekb. Comiquantititive analysis to provible with morph/motro to i oner- enalysis reconniques soon; photographs of corneas.

# Example 7: Gene-Transfer-Mediated Anglogenesis Therapy Using Truncated VRP3

Truncated VRPs are used for gene-transfer-mediated angiogenesis therapy as described, for example, in PCT/U396/02631, published September 6, 1996 as WO96/26742, hereby incorporated by reference herein in its entirety.

#### Adenoviral Constructs

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A helper independent replication deficient human adenovirus 3 system may be used for gene-transfer. A nucleic acid molecule coding for a truncated VRP subunit may be closed into the polylinker of plasmid ACCMVPLPA which contains the CMV promoter and SV40 polyadenylation signal flanked by partial adenoviral sequences from which the E1A and E1B genes (essential for viral replication) have been deleted. This plasmid is co-transferred (lipofection) into 293 cells with plasmid JM17 which contains the entire human adenoviral 5 genome with an additional 4.3 kb insert making pJM17 too large to be encapsidated. Homologous rescue recombination results in adenoviral vectors containing the transgene in the absence of E1A/E1B sequences. Although these recombinants nonreplicative in mammalian cells, they can propagate in 293 cells which have been transformed with ElA/ElB and provided these essential gene products in trans. Transfected cells are monitored for evidence of hytopathic effect which usually Noward 10-14 have sited transfection. To identify successful rucombinants, sell supernatant from places showing a cytopathic effect in prestri with protringer F (50 mg/ml with 0.5% hidium accepyl salfate and 10 mM EDTA | at 36°C for 60 minutes, phenol/ phioroform extracted and ethanol procipitated. Successful resumminants as a them inentified with FCP using grimers Builternniques (8:888-11, 1995) unprementativ to the CMV

promoter and SV40 polyadenylation sequences to amplify the truncated VRP subunit nucleic acid insert and primers (Biotechniques 15:868-72, 1993) designed to concomitantly amplify adenoviral sequences. Successful recompinants then are 5 plaque purified twice. Viral stocks are propagated in 293 cells to titers ranging between 1010 and 1012 viral particles, and are purified by double CsCl gradient centrifugation prior to use. The system used to generate recombinant adenoviruses imposed a packing limit of 5kb for transgene inserts. The 10 truncated VRP genes, driven by the CMV promoter and with the SV4C polyadenylation sequences are well within the packaging constraints. Recombinant vectors are plaque purified by standard procedures. The resulting viral vectors are propagated on 293 cells to titers in the  $10^{10}-10^{12}$  viral particles range. Cells are infected at 80% confluence and 15 harvested at 36-48 hours. After freeze-thaw cycles the cellular debris is pelleted by standard centrifugation and the by double CsCl further purified ultracentrifugation (discontinuous 1.33/1.45 CsCl gradient; 20 design prepared in 5 mM Tris, 1 mM EDTA (pH 7.8);  $90,000 \times g$  (2 hr),  $105,000 \times g$  (18 hr)). Frior to in vivo injection, the viral stocks are desalted by gel filtration through Sephanose columns such as G25 Sephadex. The resulting viral stock has a final viral titer approximately in the 1010-1010 viral particles 2.5 range. The adenoviral construct should thus be highly purified, with no wild-type (potentially replicative) virus.

## i russe ischemi: Midel Fir Anglegenesis

A lest thiracotomy is performed in isomescale pigs [3]-4] as made startly timultimes is instrumentation. Hammoni, et al. J. Clin. Towest. 20:2644-62 [1303]; Roth, et al. J. Clin. navest. 21:333-49, 1393]. Catheters are placed in the left strium and worth, provising a means to measure regional plood naw, and to measure is respected. Without are surpress on the left

atrium to permit ECG recording and atrial pacing. Finally, an ameroid constructor (ameroid), a metal ring including an ameroid substance, is placed around the proximal left circumflex coronary artery (LCx) (Hammond et al. J. Clin. Invest. 92:2644-52 (1993)). After a stable degree of ischemia develops, the treatment group receives an adenoviral construct that includes a truncated VRP gene driven by a CMV promoter. Control animals receive gene transfer with an adenoviral construct that includes a reporter gene, lacZ, driven by a CMV promoter.

Studies are initiated 35 + 3 days after ameroid placement, at a time when collateral vessel development and pacing-induced dysfunction are stable (Both, et al. Am J Physiol 253:1-11279-1288, 1387, and Roth, et al. Circulation 82:1778-89). Conscious animals are suspended in a sling and pressures from 15 the left ventricle (LV), left atrium (LA) and aorta, electrocardiogram are recorded in digital format on-line (at rest and during atrial pacing at 200 kpm). Two-dimensional and M-mode images are obtained using a Hewlett Packard ultrasound imaging system. Images are obtained from a right parasternal approach at the mid-papillary muscle level and recorded on VHS tape. Images are recorded with animals in a basal state and again during right atrial pacing (HR=200 bpm). These studies are performed one day prior to gene transfer and repeated 14 +2.5 1 days later. Rate-pressure products and left atrial pressures should be similar in both groups before and after gene transfer, indicating similar myopardial beyoden demands and liabing conditions. Edicourglwirephin measurements are made runing standardized priteria (Sahn, et al. *Dirpulacion* 59:1070, 1973). End-dinable was unickness EDWTh: and end-systolic wall thinkness (ESWTE) are measured from 5 continuous beats and averaged. Percent wall thickening (%WTh) is calculated 1 FOWTH-HEWTh)/REWTh] M 110. Cata should be analyzed without miwleade in while part the spine of an received. To

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demonstrate reproducibility of echocardicgraphic measurements, animals should be imaged on two consecutive days, showing high correlation ( $\rm r^2=0.90$ ; p=0.005).

35 ± 3 days after ameroid placement, well after ameroid closure, but performed using the contrast material (Levovist) which is injected into the left atrium during atrial pacing (200 bprn). Studies are repeated 14 ± 1 days after gene transfer. Peak contrast intensity is measured from the video images using a computer-based video analysis program (Color Vue II, Nova Microsonics, Indianapolis, Indiana), that provides an objective measure of video intensity. The contrast studies are analyzed without knowledge of which gene the animals have received.

At completion of the study, animals are anesthetized and midline thoracotomy performed. The brachycephalic artery is isplated, a canula inserted, and other great vessels ligated. The animals receive intravenous heparin (10,000 IU) and papaverine (60 mg). Potassium chloride is given to induce diastolic cardiac arrest, and the acrta cross-clamped. Saline is delivered through the brachycephalic artery cannula (120 mmHg pressure), thereby perfusing the coronary arteries. Glutaraldehyde solution (6.25%, C.1 M cacodylate buffer) was perfused (120 mmH pressure) until the heart is well fixed (10-15 min;. The heart is then removed, the beds identified using color-coded gives injected anterograde through the left anterior descending (LAD), left dircumflex (LCx), and right coronary arteries. The amercud is examined to confirm closure. Samples maker brom the normally perfused and isonemic regions are -divided into thirds and the endocardul and epicardial thirds are plastic-imbedded. Microscopic analysis to quantitate capillary number is conducted as previously described (Mathieu-Obstello, et al. Am [ Physiol 359:H204, 1990]. Four 1 µm thick transverse contions are taken film each subsamble tendocerous

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and epitardium of each region, and point-counting is used to determine capillary number per fiber number ratio at 400% magnification. Twenty to twenty-five high power fields are counted per subsample. Within each region, capillary number to fiber number rations should be similar in endocardium and epitardium so the 40-50 field per region should be averaged to provide the transmural capillary to fiber number ratio.

To establish that improved regional function and blood flow result from transgene expression, PCR and RT-PCR may be used to detect transgenic truncated VRP DNA and mRNA in myocardium from animals that have received truncated VRP gene transfer. Using a sense primer to the CMV promoter [GCAGAGCTCGTTTAGTGAAC] [SEQ I.D. NC. 41]; and an antisense primer to the internal truncated VRP subunit sequence, PCR is used to amplify the expected 500 bp fragment. Using a sense primer to the beginning of the truncated VRP subunit sequence, and an antisense primer to the internal truncated VRP sequence, RT-PCR is used to amplify the expected 400 bp fragment.

Finally, using a polyclonal antibody directed against VRP, truncated VRP expression may be demonstrated 48 hours as well as  $14\pm1$  days after gene transfer in cells and myocardium from animals that have received gene transfer with a truncated VRP gene.

The helper independent replication deficient human adenovirus E system is used to prepare transgene containing vectors. The material injected in vivo should be highly purified and contain no wild-type (replication competent adenovirus. Thus adenovirus infection and inflammatory infiltration in the nearly are minimized. By infecting the material directly into the lumen of the opposity arresty by coronary satheters, it is possible to target the gene effectively. When delivered in this manner there should be no trunsgene expression in heron bytes, and viral PNA should not

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be found in the urine at any time after intracoronary injection.

Injection of the construct (4.8 ml containing about 10<sup>11</sup> viral particles of adenovirus) is performed by injecting 2.0 ml into both the left and right coronary arteries (collateral flow to the LCx bed appeared to come from both vessels). Animals are anesthetized, and arterial access acquired via the right carotid by cut-down; a 5F Cordis sheath is then placed. A 5F Multipurpose (A2) coronary catheter is used to engage the coronary arteries. Closure of the LCx amercid is confirmed by contrast injection into the left main coronary artery. The catheter tip is then placed 1 cm within the arterial lumen so that minimal material is lost to the proximal acrta during injection. This procedure is carried out for each of the pigs.

Once gene transfer is performed, three strategies are used to establish successful incorporation and expression of the gene. (1) Some constructs may include a reporter gene (lacZ); (2) myocardium from the relevant beds is sampled, and immunoblotting is performed to quantitate the presence of truncated VRP and (3) PCR is used to detect truncated VRP mRNA and DNA.

The regional contractile function data obtained should show that control pigs snow a similar degree of pacing-induced dysfunction in the ischemic region before and  $14\pm1$  days after gene transfer. In contrast, pigs receiving truncated gene transfer should show an increase in wall thickening in the ischemic region during pacing, demonstrating that truncated VRF supunit gene transfer in abbulgance with the invention in factoristic with improved sometiment in the ischemic region with improved sometiment in the ischemic region for interpretable with improved sometiment. In the ischemic region the interventicular septiment in the normally perfused region and unaffected by gene transfer. The percent decrease in function measured by transphoracly echocardicography should be very similar to the percentage generals measured by

schemicrometry during atrial pacing in the same model (Hammond, et al. J. Clin. Invest. 92:2644, 1993), documenting the accuracy of echocardiography for the evaluation of ischemic dysfunction.

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# Séquence Listing

	:11 GENER	CAL IMPORMATION:	
5	1.2	APPLICANT:	Mollateral Therapeutics
	, ı i	TITLE OF INVENTION:	TRUNCATED VEGF-RELATED PROTEINS
10	114.	NUMBER OF SEQUENCES:	41
		EOPRESPONDENCE ADDRESS:	
15		(B) STREET:	Lyon & Lyon 533 West Fifth Street Salte 4700
		(D) STATE: (E) COUNTRY:	Los Angeles California U.S.A. 20071-2066
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20	Arg	Cys	Gly	GIY	Cys 165	Cys	Acn	Ser	Giu	Gly 176	Leu	Gl.r.	Cys	Met	Asn 175	Thr
	결원기	Thr	Jer	1;∵ 180	Len	Ser	Lys	Thr	Leu 185	Phe	Glu	Tie	Thr	Val 190	Pro	Leu
25	Ger	Gln	G1y 195	Pro	Lys	Pro	Vii	Thr 200	114	Ser	Phe	Ala	Asn 205	His	Inr	Ser
30	Суз	Arg 210	Cys	Met	Ser	lys	Leu 213	Asp	Val	Tyr	Arg	Gln 220	Val	His	Ser	Ile
	11.5 225	Arg	Arg	Ser	Leu	Ero 230	Ala	Thr	Lest	Fro	31m 235	Суз	Gln	Λια	Ala	Asn 240
3.5	Lys	Thr	Cys	Pro	Thr 245	Asn.	Tyr	Met	Trp	Asn 250	Aar.	His	ΙΞē	Cys	Arg 255	Cys
	Leu	Ala	Gln	GLu 260	Asp	Phe	Met	Phe	Ser 265	Ser	Asp	Ala	Gly	Asp 270	Asp	Ser
4 C	Thr	Asp	31. G1.	Ph∙e	His	Aup	110	Cys 280	بنين	Erc	Asn	Lys	31d 235	Leu	Asp	Glu
4.5	94.i	Thr 290	Cys	Sin	Сув	Val.	Cys 295	Arj	Åi	Sly	Lau	Arg 300	Pro	Ala	Ser	Сув
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23	Asn	Gly	Ser 35	Ser	Glu	Mail	Glu	Val 40	Val	Pro	Phe	Gln	Glu 45	Val	Trp	Gly
30	Arq	36r 30	Tyr	Cys	Arg	Pro	11e 55	Glu	Thr	Leu	Val	Asp 60	Ile	Phe	Gln	Glu
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35	전숙되	Arg	Cys	317	Gly 85	Cys	Tya	Asn	Asp	31u 90	Jly	Let	Glu	Cys	751 95	Pro
40	Thr	31/2	Glu	Ser 100	Asn	Val	Thr	Met	Gln 105	11e	Met	Arg	Ile	Lys 110	Pro	His
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10	Fro	Ala	Gln	Ala 20	Pro	Val	Jer	Gln	250 25	Asp	Ala	Pro	Gly	His 30	Gln	Arg
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20	Cys	Pro	Asp	Asp	Gly 85	Leu	Glu	Cys	Val	90 90	Thr	Gly	Gln	His	Gin 95	Val
25	Arş	Met	Gln	Ile 100	Leu	Met	Ile	Arq	Tyr 105	Pro	Ser	Ser	Gln	144 110	Gly	Glu
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30	Asp	Ser 130	Ala	Val	Lys	Gln	Asp 135	Arg	Ala	Al a	Thr	Fro 140	His	His	Arg	Pro
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15	Thr	Gla	Glu	Val	Asn 35	Val	Thr	Met	Glu	Lau 90	Leu	Gly	Ala	Ser	Gly 95	Ser
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35	Met 1 Met	(ti) (xi) Lys Tyr	(A) (B) (D) MO: SEC Leu	LECUI Thr Thr	ENGT! YPE: DPOL: LE T' CE D. Ala S	H: OGY: YPE: ESCR Thr	IPTI Leu Cys	Gin	3800 : Val Ser 23	amino line Prote ID No V_1 10	o ac: ar ein C: T Val	td 7: Ala Asn	Leu Asp	Ser 30	15 Pro	Pro
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35 40 45	Met 1 Met Ser	(ti) (xi) Lys Tyr Thr	(A) (B) (D) MO: DEC Led Asn Asn 35 Thr	LECUI DUENT Thr Leu Asp Val	ENGTHYPE: SPECIAL STREET FROM Trp Val  Corr Corr Corr Corr Corr Corr Corr Co	H: OGY: YPE: ESCR Thr Gli Met Tyr Ard	Led Cys Arg	Gin Val Thr 40 Gly Val	Ser 23 Leu Str	amino line Prote Prot Vil 10 Gin Asp Gil Ali  Jai	o according to a control of the cont	Asn Ser Pro ()	Leu Asp Gly 45 Glu Thr	Ser 30 Cys Ser Art	Pro Lys The	Pro Pro Asr

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25	Met	Gly	Asr. 35	Val	Val	Lys	Gln	Leu 40	Val	Pro	Ser	Cys	Vа 45	Thr	Val	Gln
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	Gly 65	Gln	His	Gln	Val	Arg 70	Net	Gln	Ile	Leu	Met 75	Ile	Gln	Tyr	Pro	Ser 8)
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40	ysą	Pro	Lys	Lys 100	Lys	31::	Ser	Ala	Val 105	Lys	Pro	Азр	Ser	Pro 110	Arg	Ile
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45	Cys	Arg 130	Cys	Arg	Cys	Arţ	Ar; 335	Ary	Arg	Pne	Leu	His 140	Cys	31n	Gly	Arg
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20	Glu	Ser	Ala	Val 100	Lys	Pro	Asp	ger	103	Arq	110	Leu	Cys	Pro 110	Pro	Суз
25	Thr	Gin	Arg 115	Arg	Gln	Arg	Pro	Asp 120	Fro	Arg	Thr	Cys	Arq 125	Cys	Arg	Cys
	Arg	Arg 130	Arg	Arg	Phe	Leu	His 135	Cys	Gln	Gly	Arg	Gly 140	Leu	Glu	Leu	Asn
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	Ser	Cys	Val 35	Thr	Val	Gln	Arg	Cys 40	Gly	Gly	Cys	Cys	Pro 45	Asp	Asp	Gly
35	Leu	Glu 50	Cys	Val	Pro	Thr	Gly 55	Gln	His	Gln	Val	eo Yzd	Met	Gln	Ile	Leu
40	Met €5	ile	Gln	Tyr	Pro	Ser 70	Ser	Gln	Leu	Gly	Glu 75	Met	Ser	Leu	Glu	Glu 8C
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4.5	Pro	Asp	Ser	Pro 130	Arg	Ilb	Lou	Cys	Pro 105		Cys	Thr	Gln	Arg 110	Arg	Gln
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20	Tyr 63		Jer	Ser	Sin	Leu 70	Sly	Glu	Met	Ser	Leu 75	Glu	Glu	His	Ser	Glr 3C
20	Cyn	Glu	lys	Arq	Pro 85	Lys	Lys	Lys	Gli	Ser 90	Alā	Val	Lys	Pro	Asp 35	Ser
25	Pro	Arg	Ile	Leu 100	Сув	Pro	Pro	Cys	Thr 105	Gla	Arg	Arg	Gln	Arg 110	Pro	Asp
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1.C	Tr.r	lys	Ar; 115	<sup>er</sup> ys	Arg	Oys	Arı	Arg L20	Arg	Arg	Phe	leu	Eis 125	Cys	Gln	Gly
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3.5	Mes	Gly	Thr 35	Val.	Ala	Lys	Gln	Leu 40	Val	Fro	Ser	Cys	Val 45	Thr	Val	Gln
	Arg	Cys 50	G y	dly	Суз	Cys	Pro 55	Asp	Asp	Ģlу	Leu	Glu 60	Cys	Val	Pro	Thr
40	31y 65	Glr.	45	Gln	Val	Arg 20	Het	Gln	Ile	Leu	Met 75	Ile	Arg	Tyr	Pro	Ser 80
45	Sor	Gin	Lėu	Sly	61u 85	Met	Sor	Leu	Slu	51 : 90	His	Ser	Gir	Cys	Gla 95	Cys
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	(3) 109	GRMATIAN FOR J	SECTION OF D	15:	
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5		(A) LENGTH: (B) TYPE: (D) TOPPLOS		173 amine acid amine acid linear	s
10	(ii)	MOLECULE TYP	PE:	Protein	
. U	(X1,	SEQUENCE DES	SCRIPTION: SE	EQ ID NO: 15:	
15	Lys Vi. l	Val Ser Trp :	lle Amp Val 1	Tyr Thr Arg Ala T 10	hr Cyd Gln Pro 15
	Ara Gli	Val Val Val I 20		Val Olu Leu Mer G 25	ly Thr Val Ala 30
20	Lys Gln	Len Val Pro 3 35	Ser Cys Val 1 40	The Val Gin Arg C 4	
	Cys Pro 50	Asp Asp Gly I	Led Glu Cys 1 55	Val Pro Thr Gly G 60	ln His Gln Val
25	Arg Met 65		Met lle Arg 1 70	Tyr Fro Ser Ser G 75	in Leu Gly Glu 80
30	Met Ser	Leu Gla Glu : 85	His Ser Gln (	Cys Clu Cys Arg P 90	ro Lys Lys Asp 95
	Sar Ala	Val Lys Pro : 100		Alu The Peo His H 105	is Ard Pro Gln 110
35	Pro Ar:	Ser Val Pro : 115	Gly Trp Asp : 120	Ser Ala Pro Gly A 1	la Pro Ser Pro 25
	Ala Asp 130		Pro Thr Pro A	Ala Pro Gly Pro S 140	er Ala Eis Ala
40	Ala Pro 145		Ser Ala Leu ' 150	Thr Pro Gly Pro A 135	la Ala Ala Ala 160
45	Ala Asp	Ala Ala Ala . 165	Ser Jes Val .	Ala Lys Gly Gly A 170	la
40	(L) INF	GRMATION FOR	SEQ ID NO:	16:	
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Sec Dys Val Thr Val Tin Arg Dys Gly Gly Dys Cys Pro Asp Asp Gly 35  $$40^{\circ}$$ Len 31: Gyb Val Bro Thr Gly Gin Hib Gin Val Arg Met Gin Ile Leu 50 60 Men lie Arg Tyr Fro Ser Ser Gin Leu Gly Glu Met Ser Leu Glu e5 70 75 10 His Ser Jim Cys Glu Cys Arg Pro Lys Lys Asp Ser Ala Val Lys Pro Asp Arg Ala Ala The Pro His His Arg Pro Gle Pro Arg Ser Val Pro Gly Trp Asp Ser Ala Pro Gly Ala Pro Ser Pro Ala Asp Ile Thr His 115 120 125 Pro Tor Pro Ala Pro Gly Pro Ser Ala His Ala Ala Pro Ser Thr Thr 130 140 Ser Ala Len The Pro Gly Pro Ala Ala Ala Ala Ala Asp Ala Ala Ala 145 \$150\$25 Ser Ser Val Ala Lys Gly Gly Ala 165 (2) INFORMATION FOR SEQ ID NO: 17: 30 (1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 163 amino acids (B) TYPE: amino acid 35 TOPOLOGY: linear (ii) MOLECULE TYPE: (x1) SEQUENCE DESCRIPTION: JEQ ID NO: 17: 40 Arg Ala The Cys Gin Pro Arg Gl: Val Vil Val Pro Lou The Val Glu Lou Met Gly Thr Val Ala Lys Gin Le: Val Pro Ser Cys Val Thr Vai 45 The TV has \$2 to 5 to 12 terms of the general literature by d Part Lyn, Lyn Rig (1976 A d Da ) Igna End Awa And A d Agadhan 95 95 TO MAIN BY ALL THE WAR THE ATTEMPT OF MAIN TOP MAY TAPE AND THE ATTEMPT OF THE AT

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30	Met	Gly	Thr 35	Val	Ala	Lys	Gln	Leu 40	Val	Pro	Ser	Cys	Val 45	Thr	Val	Gln
	Arg	Cys 30	Sly	Gly	Зуs	Cys	Fro 55	Asp	Asp	Gly	Leu	Glu 60	Cys	Val	Pro	Thr
3.5	G_Y 65	Gln	His	Gln	Val	Arg 70	Met	Gln	ils	Leu	Met 75	Ile	Arg	Tyr	Pro	Ser 80
40	Ser	Gln	Leg	Gly	31u 85	Met	Ser	Leu	311	Gln 30	Fis	Ser	Gin	Cys	Glu 95	Cys
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45	Fro	HLS	Hls 114	Arq	Fro	Gln	Princip	Arg 120	ser	73.	Fro	(G) Y	7rp 125	qsA	der	Ala
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15	Arg 310	741 Val 20	Val Ero	Led Thr	Val 25	. Glu	Leu	Met	Sly	Thr 30	Val	Ala
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20	Oya Pro 50	Asp Asp	Gly Leu	Glu Oya 55	Va1	. Ero	Thr	Gly 60	Jln	His	Gln	Val
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3.0	Asp Ser	Ala Val 100	Lys Gln	Asp Arg	Ala 105		Thr	Fro	His	His 110	Arg	Pro
	Gin Pro	Arg Ser 115	Val Pro	Gly Trp 120	Asp	Ser	Ala	Pro	Gly 125	Ala	Pro	Ser
3.5	Pro Ala 130	Asp Ile	Thr 31n	Ser His 136	£e:	Ser	lro	Arg 140	Pro	Leu	Сув	Pro
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	Ser	Fhe	Беш	Arg	Cys 165	Gln	Giy	Arg	Gly	Leu 170	Glu	Leu	Asn	Pro	Asp 175	Thr
30	Cys	Arg	Суз	Arg 190		Leu	Arg	Arg								
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1.5	din	J., y	Ara	Cly	165	Glu	Les	Äsn	Pro	Азр 170	Thr	Cys	Arg	Cys	Arg 175	Lys
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30	His :	Tyr	Asn	Thr	Glu 5	Ile	Leu	Lys	Ser	110 10	Asp	Ash	Glu	Trp	Arg 15	Lys
35	Tar	Gir.	Сув	Met 20	Pro	Arg	014	Val	Cys 25	Ile	Asp	Val	Gly	Lys 30	Glu	Ph
	σιy	Va L	A1a 35	Thr	Asn	Thr	F'h÷	Phe 40	Ьγз	Fro	Fro	Cys	Val 45	Ser	Val	Ту
40	Arg	Cys 50	Gly	Gly	Cys	Суз	Asn 55	Ser	Glu	Gly	Leu	Gln 60	Cys	Met	Asn	Th
	3es 68	Thr	Ser	Tyr	Leu	Ser 70	Lys	Thr	Lou	Pr.e	Glu 75	Ile	Thr	Val	Pro	Let 80
4.5	Ser	Gln	Cly	Pro	Lys 35	Pro	Val		Ile	3er 90	Phe	Ala	Aan	Hls	Thr 95	Se.
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	7-3	T 11	'ys	31 n 13 c	Cys.	ā	2∵s	Ard	Ala 185	Gly	Leu	yzş	Pro	Ala 190	Ser	Cys
5	31.y	Pro	313 195	Lys	Glu	Leu	Asp	Arg 200	Asn	Ser	Cys	Gln	Cys 205	Val	Cys	Lys
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10	Asr. 228	The	Cys	Oln	Cys	Val 230	Jys	Lys	Vil	Thr	фув 235	Pro	Arg	Asn	Gln	Pro 240
15	Ţŧu	Ach	Pic	3 - 7	Lys 245	Сун	Ala	lys	3212	Cys 250	Thr	Glu	Jer	Fro	Gln 255	Lys
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20	Λrq	Vad	Pro 275	Суз	Thr	Asa	Arg	Gln 280	Lys	Ala	Cys	Glu	Pro 285	Gly	Fhe	Ser
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40 45	Tie Ard Thr Cos Sec	(Mi) Leu Glu Fre Ass	(B) (C) MCI SE( Lys Val Pne 35 Ver Tor	Cys 20 Lys Lys Lys Lys Lys Lys	YPE: OPOLITED THE TOTAL DESCRIPTION THE DESCRI	OGY: YPE: ESCR Asp Asp Dec: Control To	Ash Val Cys Lit	Gla Gly Vai 40 -71 The	SEQ Trp	emined lines Protected Argument 10 Mc Giu 741 Argument 10 Argument	Description of the control of the co	Ed 23: Thr Gly Arg	Gin Val Cys 45 thr sin	Ala 30 Gly Wer	The Gly .ve	Ash Cys Lyu Sy Say

The Went Fine The Bar App Ala City Asp App Jer Thr App 119 Phe Bio 148   148   155   155   155   155   165   165   165   165   155   155   155   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165   165		AJI.	772 130	Mut	7.25	Asn	Aun	H:s 135	110	Пуа	Аст	lys	Let 140	Ala	Sin	شاذ	Ąsp
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186	10	Азр	110	Cys	Gly	Pro 165	Asr.	Lys	314	Leu	43p	Glu	Glu	Thr	Суз		Cys
Let   Asp   Arg   Arg   Arg   Ser   Cyr   Cln   Cyr   Val   Cyr   Lyr   Arg   Lyr   Let   She   Pro   200	- 5	Val	Cys	Arg	A_a 130	Gly	Leu	Arq	Pro			Cys	Gly	Pro		Lys	Glu
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25	20	Ser	Gin 219	975	43-7	Ala	Asn	Arg 215	Sin	Phe	Asp	319		Thr	Cys	Gln	Cys
245   250   255   255   367   377   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378   378				Lys	Arq	Thr		Pro	Arg	Asn	din		Leu	Asn	Pro	З1у	
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275   280   295   295   35   35   35   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   375   3	30	Lys	Lys	Phe		His	Gln	Thr	Суз		Cys	Tyr	Arg	Arş		Суз	Thr
35 236 295 300  (27 INFORMATION FOR SEC 10 NJ: 24:  (11 SEQUENCE CHARACTERISTICS:  (A1 LENGTH: 29T amino acids amino acids (B) TYPE: amino acids amino acid linear  (A2 CENGTH: 29T amino acid linear  (A3 CENGTH: 29T amino acids amino acids (B) TYPE: Protein  (A1 ACID TOPOLOGY: Protein  (A2 ACID TOPOLOGY: Protein  (A2 ACID TOPOLOGY: Protein  (A3 ACID TOPOLOGY: Protein  (A4 ACID TOPOLOGY: Protein  (A5 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A7 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A6 ACID TOPOLOGY: Protein  (A7 ACID TOPOLOGY: Prote		Asn	Ang		Lys	Ala	Cys	Glu		Gly	Phe	Ser	Tyr		Glu	Sla	Val
43  (ii SEQUENCE CHARACTERISTICS:  (A) LENGTH: Common acids amino acids amino acids amino acids amino acids amino acid (E) TYPE: Protein  (XI) SEQUENCE TYPE: Protein  (XI) SEQUENCE DESCRIPTION: DEC TD NC: 24:  A: A: A: D: Ti. In: Ar:	3.5	Oys		Cys	Val	Pro	Jer		jin	īvs	Arj	970		Met	Ser		
40  (A* LENGTH: 29" amino acids amino acids amino acids amino acids amino acid (B) TYPE: amino acid linear  45  (1) TOPOLOGY: Protein  (xi) OEDUENCE DESCRIPTION: OEO TD No: 24:  A: A		129		OFMA.	FION	FCR	SEÇ	10 1	:	24:							
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SF Pro Dyn Mar Den Mar Tor Dr. The Ser Tyr Lei Ser Lys Thr Lou Phe			(RI)	SE	DUEN	DE DI	ESCRI	ITTE	D14: 1	PEQ	ID W	: :	24:				
Fig. 190 Mai Jos Wal Tur Dri Jos Bly Sly 190 Mas Der Slu Sly 35 45 45 Tur Lou Phens 190 Mai Jos Wei Ash Tur Dri Ser Tur Lei Ser Lys Tur Lou Phens 190 Mai	i.	A 1		11.	1::	Al f	9	11.1	. i.	** : ·	e ·	î	ver i	ly i	. : -	::::	. 1.4
180 - Louis Med Ago The Page The Ger Tyr Les Sor Lys The Less Phé		- 1 - <b>i</b> -		ÿ., ŗ	- 1		F *	12			Ť.		772	i ine	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	1 y s	1::-
	5 6	₽:∋	lyw	Па. 35	ជូក វា	Val	Tor	.127	Uma Ak	17	Яy	iys	wys	Asn 45	Jer	31u	ilv
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	r'n4	A_a	M3D	313	Fnr 85	Ser	Cys	Arg	Cys	Met 90	Ser	Lys	Leu	Asp	Val 35	Tyr
5	Arj	Gla	7.41	H13 100	Ser	lie	11.9	Ary	Arg 105	Ser	Leu	Pro	Ala	Thr 113	Leu	Pro
10	3	tys	G.n 115	Ala	Ala	Asn	Lya	Thr 120	Cys	Pro	Thr	Aar.	Tyr 125	Met	Trp	Asn
	Åse fi	His 130	114	772	Arş	iys	£æu 1.:5	Ala	Iln	314	Asp	2ne 140	Met	Pne	Ser	Ser
15	Asp 145	Als	Gly	Aar	Авр	Ser 150	Phr	Asp	Sly	Ph++	His 155	quA	Tie	Cys	З1у	Pro 160
	Asn	Lys	Glu	Leu	Asp 165	Glu	Slu	Thr	Cys	Gln 170	Cys	Val	Суз	Arg	Ala 175	Gly
26	Leu	Arg	Pro	Aia 130	Jer	Cys	Gly	Ero	Н±З 185	Lys	Glu	Leu	qzA	Arg 190	Asn	Ser
25	ùys	Gin	178 195	Val	Cys	Lys	ASD.	шуз 200		the	gro	ser	31n 205	Cys	Gly	Āla
	Ash	Arg 210	Glu	Phe	Asp	Glu	Asn 215	Thr	Cys	31::	Cys	Val 220	Cys	Lys	Arg	Thr
30	Оув 225	Pro	Arg	Asn	Gln	Pro 230	Leu	Asn	Pro	Glv	Lys 235	Суз	Ala	Суѕ	Glu	Cys 240
	Thr	Glu	Şer	Pro	Gln 243	Lys	Cys	Les	Leu	Lys 250	Gly	Lys	Lys	Phe	His 255	His
35	Gir.	Thr	Cys	3er 260	Cys	Tyr	VL-1	Arg	255 255	Cys	Thr	Asn	Arg	Gln 270	Lys	Ala
40	Cys	Glu	Pro 275	Gly	Phe	Ser	lyr	Ser 230	Glu	Sid	Val	CAs	Arg 285	Cys	Val	Pro
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5	The	3 <b>er</b> 50	Thr	30%	ryr	Leu	Ser 55	Lyc	Thr	Leu	Phe	G1u 60	Ile	Thr	Val	Pro
,	1.5u 65	Jor	31n	Gly	Pro-	Lys 70	Fro	Val	Thr	Ile	Ser 75	Phe	A1.4	Asn	His	Thr 80
10	Jer	Зүз	Arş	Cys	Met 35	Ser	Lya	Leu	Asp	7a1 90	Tyr	Arg	Glm	Val	His 95	Ser
		119	Arş	Ara 100	395	Lea	Pro	Ala	Thr 105	Leu	Pro	Gln	Cys	Gln 110	Ala	Ala
15	Asn	Lyš	Thr 115	Cys	Pro	Thr	Asn	177 120	Met	Trp	Adn	Asn	His 125	Ile	Cys	Arg
20	Суз	Leu 130	Ala	Glm	Glu	Asp	Phe 135	Met	Phe	Ser	Ser	Asp 140	Ala	GTA	Asp	Asp
	Ser 145		Asp	Gly	Phe	Hls 130	Asp	Ile	Cys	gly	Pro 155	Asn	Lys	Glu	Leu	Asp 160
25	Glu	Glu	Thr	Суз	31n 165	Cys	Val	Cys	Arg	Ala 170	Gly	Leu	Arg	Pro	Ala 175	Ser
	Cys	Gly	Prc	His 190	Lys	GLu	Leu	Азр	Arg 185	Asr.	Ser	Cys	Gln	Cys 190	Val	Cys
30	Lys	Asn	Lys 195	Leu	Phe	Pro	Ser	Gln 200	Суз	Gly	Ala	Asn	Arg 205	Glu	Phe	Asp
35	Glu	Asn 210		Cys	Gln	Cys	Val 215	Cys	Lys	Arg	Thr	Cys 220	Pro	Arg	Asn	Gln
	Pro 225		Asr.	Pro	Gly	Lys 230		Ala	Cys	Glu	0ys 235	Th.	Gla	Ser	Pro	Gln 240
40	Lys	Cys	Leu	Leu	Lys 245	Gly	Lys	Lys	Phe	His 250	His	Gln	Thr	Cys	Ser 255	Cys
	Tyr	Arg	Arg	Fr: 260	Cys	Thr	Adn	Arq	Gln 265	Lys	Ala	Cys	Glu	Pro 270	Gly	Phe
45	Ser	Tyr	Ser 275		Slu	741	Cys	Arg 280		Val	Pro	Ser	Tyr 285		Lys	Arg
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		٠	, <sup>7</sup> **.	1 1711	} ~	EAP N	. 11.7.7		+							
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	De 12	Aan	Ala	qsA	201 0	Asn	Thr	Lya	317	Trp	305	Glu	Val	Leu	Lys 15	Gly
5	Cer	311	Cys	Lys 10	Pro	Arg	Pr-	119	Val 25	Val	Pro	Val	Ser	Glu 30	Thr	His
	£ra	314	Leu 35	Thr	Sec	Gln	Arq	Pne 40	Asn	Pro	Sro	Cys	Val 45	Thr	Leu	Met
10	Arş	Cys 50	ЭГА	Gly	Cvs	Cys	Aan 35	Aup	Glu	Sor	Lau	31u 60	Cys	Val	Pro	Thr
15	Ģ1-1 65	gin	Val	Asn	V.11	Thr	Mos	11*1	Len	Leu	9.7	Ala	Ser	Gly	Ser	31y 80
	Jur	Asn	Gly	Met	Gin 85	Arj	Let	Ser	Phe	Val 90	Glu	His	Lys	Lys	Cys 95	Asp
20	Суз	Ara	Pro	Arg 100	2ne	Thr	Thr	Thr	Pro 105	Pro	Thr	Thr	Thr	Arg 110	Pro	Pro
	Arg	Arg	Arg 115	Arş												
25																
	(=)	INFO	DRMAT	CICN	FOR	SEQ	ID:	: 02	27:							
30		; Ť.,	SE	QUENC	CE CI	HARAG	CTER:	ETIC	CS:							
50			(A) (B		ENGTI YPE:	H:				111 a	amin		ids			
			(0)			DGY:				linea						
35		(11)	(0)	T (							ar					
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35 40		(xi)	(D) MO:	TO LECUI QUENO	DE DI	YPE: ESCR	IPTI:		i Seç :	linea Prote	ar ein	27:	5112	Суз	Lys 15	Pro
	Asn 1	(xi) Thr	MO: SEC	T( LECU QUEN Gly	Trp 3	YPE: ESCR Ser	IPTI: Glu	722	i BEQ : Leu	Prote Prote ID NO Lys 10	ar ein D:	27: Ser			15	
	Asn 1 Arj	(xi) Thr Pro	(D) MOI SEC Lys	TO LECU QUEN Gly Val 20	OPOLK DE DI Trp 5	YBE: ESCR Ser Ser	IPTIC Glu Val	VxL Ger	3 <b>5</b> S <b>EQ</b> : Let Siu Si	Line: Prote ID NO Lys 10	ar ein D: Gly His	27: Ser Pro	Glu	Leu 30	15 Thr	Ser
40 45	Asn 1 Ar;	(xi) Thr Pro Arg	(D) MO: SEC Lys I.e	TOULECUI QUENG Gly Val 20 Aso	OPOLK  LE TI  CE DI  Trp  5  Val	YPE: ESCR Ser Pro	IPTI: Glu Val Cys	Val Ser Val 40	SEQ : Let Sid Sid Shr	Prote Prote ID NO Lys 10 Thr	ein O: Gly His	27: Ser Pro Arg	Glu Cys 45	Leu 30 Gly	15 Thr Gly	Sor
40	Asn 1 Ar; Gln	(xi) Thr Pro Arg Act	MO: SEC Lys Tie The 35	TO LECU QUEN Giy Val 20 Asn	DE DI Trp 5 Val	YPE: ESCR Ser Pro Pro	IPTIC Glu Val Cys	Val Ser Val 40	EEQ : Let  Sid  Sid  Thr	Prote Prote ID NO Lys 10 Thr Leu	ein O: Gly His Met	27: Ser Pro Arg	Glu Cys 45	Leu 30 Gly	Thr Gly Acr	Ser Cys Val
40 45	Asn 1 Arg Gla	(xi) Thr Pro Arg Acc. SC	MOD SEC Lys Tie The 35 Amp	TO T	DE DI Trp 5 Val Pro	YPE: ESCR Ser Pro Lo:	IPTIC Glu Val Cys	Val Ger Val 40	EEQ : Let  Sid  Sid  Thr	Prote Prote Lys 10 Thr Leu Ter	ein C: Gly His Met	27: Ser Pro Arg 31: 4:	Glu Cys 45 Wha	Leu 30 Gly	Thr Gly Acr	Ser Cys Val
46 45 50	Agn 1 Arj Gln Gln Gln	(xi) Thr Pro Arg Act. 50 Men	MOD SEC Lys Tie The 35 Amp	TOURSE	DPOLK LE TI DE DI Trp b Val Pro Con Pro	YPE: ESCR Ser Pro Lui	Cys  Cys  Ala	Val Ser Val 40	SEQ :	Prote Prote ID NO Lys 10 Thr Leu Tec 200	ar ein C: Gly His Met Thr	27: Ser Pro Arg 31: 4:	Glu Cys 45 Win Arm	Leu 30 Gly	Thr Gly Acr Met Arg	Ser Cys Val

5		(E,	LENGTH: TYPE: TOPOLOGY:			amino ac: c acid ar	ias	
-	(ii)	MOLEC.	ULE TYPE:		Prot	ein		
	(x:]	SEQUE	NGE DESCR	IPTION: S	HEQ ID N	0: 28:		
10	ber Glu 1	Val Le	i Lys Gly 5	Ser Glu	Cys Lys 10	Pro Arg	Pro Ile	Val Val 15
<u> 15</u>	Pro Val	Ser SP 2)	u Thr His	Pro Glu	Leu Thr 25	Ser Gln	Arg Ehe 30	Asn Pro
10	Pro Cys	Val Th 35	r Lou Met	Arg Cys 40	Gly Gly	Cys Cys	Asn Asp 45	Glu Ser
20	Deu Glu EC	Cys Ya	l Pro Tar	014 Gla 55	Val Aan	Val Thr 60	Met Glu	Leu Leu
	Gly Ala 65	Ser Gl	y Ser Gly 70	· Jer Ain	Gly Met	Gln Arg 75	Leu Ser	Phe Val
25	Glu His	Lys Ly	s Cys Asp 85	) Tys Arg	Pro Arg 90	Phe Thr	Thr Thr	Pro Pro 95
30	The Thr	Thr An		Arg Arg	Arg Arg 105			
33	:2 INF	ORMATIO	N FOR SEQ	II NC:	23;			
	(i)	SEQUE	NCE CHAPA	CTERISTI:	CS:			
35		(B)	LENGTH: TYPE: TOPOLOGY:			amino ac o acid ar	ıds	
4.0	(11)	MOLEC	CLE TYPE:		Frot	o i n		
40	(Ri				2.7.7.7	OIII		
	,	SEÇUE	NCE DESCR	RIPTION: .				
45				RISTION: . o Arg Pro	SEO ID :	O: 29:	Val Ser	Glu Thr 15
45	Gly Ser 1	Glu Cy	s Lys Pro 5 u Thy Sei		SEO ID :   Ile Yal   10	O: 29: Val Pro		15
45	Gly Ser 1	Glu Cy Glu Le	s Lys Pro 5 u Thr Sei	o Arg Pro	SEQ ID t The Val 10 Phe Asr 25	0: 29: Val Pro	Cys Val	15 Thr Leu
45 5	Gly Ser 1 His Pro Mar Arg	Glu Cy Glu Le Clu Le	s Lys Pro 5 u The Ser v My dv.	o Arg Pro	SEQ ID : Ile Val 13 Phe Asr 25	O: 29: Val Pro Fro Pro	Cys Wal 30 -011 Cyr 45	Thr Leu Vul Pr
45 55	Gly Ser 1 Bis Pro Mon Arit The Single Gly Tos 31	Glu Cy Glu Le gr Typ d. Yer Tr	s Lys Pro 5 u The Sec y May de. 1 Ach Tel y Mer 111	o Arg Pro	SEQ ID :  Ile Val 10  Phe Asr 25  Asr No.  Sec She	O: 29: Val Pro Pro Pro Lea G.y Val Gla	Cys Val 30 GL: Cys 40 Al: Scr H:3 Lyc	Thr Leu Vul fro Cly Jon Clys Cys 60
7,7	Gly Ser 1 Bis Pro Mon Arit The Single Gly Tos 31	Glu Cy Glu Le gr Typ d. Yer Tr	s Lys Pro 5 u The Sec y May de. 1 Ach Tel y Mer 111	o Arg Pro	SEQ ID :  Ile Val 10  Phe Asr 25  Asr No.  Sec She	O: 29: Val Pro Pro Pro Lea G.y Val Gla	Cys Val 30 GL: Cys 40 Al: Scr H:3 Lyc	Thr Leu Vul fro Cly Jon Clys Cys 60

	( -)	INFO	I AMA	MCIN	FOR	SEQ	i II	l'.:	30:							
5		( - 1	350	JUENC	Œ CI	IARAC	CTERI	OTIC	23:							
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10		111	MOI	LECUI	E TY	PE:			1	Prope	ein					
		(x:)	JEÇ	MENC	DE DE	ESCRI	EPTIC	M: J	SEQ.	ID NO	): :	31):				
15	Asr. 1	Aup	.iqr	Pra	Pro 5	Jer	inr	Asn	Asp	1rp 10	Met	Aru	Thr	leu	Asp 15	Lys
	Ser	Gly	Cys	Lys 20	Pro	Arg	Asp	Thr	Val 25	Val	Tyr	Leu	Gly	G_u 30	Glu	Tyr
20	Pro	Glu	3er 35	Thr	Asn	Leu	Gln	Tyr 40	Asn	Pro	Arg	Cys	Val 45	Thr	Val	Lys
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10	Ser	Val	Thr	Glu	His 85	Thr	Lys	Cys	Asp	Cys 90	lle	Gly	Arg	Thr	Thr 95	Thr
15	Thr	Pro	Thr	Thr 100	Thr	Arg	Glu	Pro	Arg 105	Arg						
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35	Val	Pro	1.eu 35	Ser	Met	Glu	Leu	Met 40	Gly	Asn	Val.	Val	Lys 45	Gin	Leu	Val
40	Pro	Ser 50	Cys	Val	Thr	Val	Gln 55	Arg	Cys	Gly	Sly	Cys 60	Суз	Pro	Asp	Asp
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25	Gly Leu 65	Glu Cys	Val Pro 70	Thr	Gly	Gln	His	Gln 75	Val	Arg	Met	Gln	Ile 80
	leu Met	Ile Arg	Tyr Pro 85	Ser	Ser	Gln	Leu 90	Gly	Glu	Met	Ser	Leu 95	Glu
30	Glu His	Ser Gln 100	Cys Glu	Cys	Arq	Pro 105		Lys	Asp		Ala 110	Val	Lys
35	Pro Asp	Arg Ala 115	Ala Thr		His 120	His	Arg	Pro	Gin	Pro 125	Arg	Ser	Val
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40	His Pro 145	Thr Pro	Ala Pro 150		Pro	Ser	Ala	His 155	Ala	Ala	Pro	Ser	Thr 160
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35	Asp	Cys	Arg 115	Pro	Arg	Phe		Thr 120	Tnr	Pro	Pro	Thr	Thr 125	Thr	Arg	Pro
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Ard Asp Thr Val Val Tyr Leu Gly 31d Glu Tyr Pro Glu Ser Thr Asn 50 60 Leu Gin Tyr Asn Pro Arg Cys Val Thr Val Lys Arg Cys Ser Gly Cys 63 75 80 5 Cys Asn Gly Asp Gly Glm Ile Cys Thr Ala Val Glu Thr Arg Asn Thr 10 Thr Val Thr Val Ser Val Thr Gly Val Ser Ser Ser Ser Gly Thr Asn 105 Ser Gly Val Ser Thr Asn Leu Gln Arg Ile Ser Val Thr Glu His Thr 15 Lys Cys Asp Cys Ile Gly Arg Thr Thr Thr Thr Pro Thr Thr Thr Arg 135 Glu Pro Arg Arg 20 145 (2 INFORMATION FOR SEQ ID NO: 40: (1) SEQUENCE CHARACTERISTICS: 25 (A) LENGTH:
(B) TYPE: 26 amino acids amino acid (D) TOPOLOGY: linear 30 (ii) MOLECULE TYPE: Protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40: Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu  $1 \hspace{1cm} 5 \hspace{1cm} 15$ 35 Tyr Leu His His Ala Lys Trp Ser Gln Ala 20 40 (2) INFORMATION FOR SEQ ID NO: 41: (i) SEQUENCE CHARACTERISTICS: 20 base pairs (A) LENGTH: (B) TYPE:
(C) STRANDEDNESS:
(D) TOPOLOGY: 45 nucleic acid single linear (x4) CEQUENCE DESCRIPTION: SEC ID No: 40: 5.0 SCAGAGCICO TITAGIGAAC

## <u>Claims</u>

- A truncated VRP subunit having a deletion of at least one of the amino acid residues N-terminal to the first cysteine of the core sequence of said subunit.
  - 2. The truncated VRP subunit of claim 1 wherein the VPP is a human VRP.
- 3. The truncated VRP subunit of claim 1 wherein said VFP is selected from the group consisting of VEGF-B, VRF-2, VEGF-C, PIGF, VEGF-3, powvirus CPF-1, and powvirus ORF-2.
- 4. The truncated VRP subunit of claim 1 wherein said VPP is VEGF-B.
  - 5. The truncated VRP subunit of claim 1 wherein said VRP subunit comprises an amino acid sequence of Figure 2.
- 20 6. The truncated VRP subunit of claim 1 wherein the amino acid residues N-terminal to the first dysteine of the core sequence of said subunit are deleted.
- 7. The truncated VRF subunit of claim 1 wherein the 25 amino acid sequence N-terminal to said core sequence comprises 2 to 5 amino acid residues.
  - 5. The truncated VFF subunit of claim " wherein duid in the small estimated to the times time duming acid residues immediately N-terminal to the first cysteine of the core sequence of said VRP subunit.
    - Fig. The truncated VRT sumunit of claim 1 wherein the unit of all sequence Setterminal to haid for he picace comprises

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- 10. The truncated VRF supunit of claim 1 wherein said 6 to 10 amino acid residues comprise 6 to 10 of the consecutive amino acid residues immediately N-terminal to the first systeine of the core sequence of said VRF subunit.
- 11. The truncated VRP subunit of claim 1 wherein the amino acid sequence N-terminal to said core sequence comprises 11 to 20 amino acid residues.
- 12. The truncated VRP subunit of claim 1 wherein said 11 to 20 amino acid residues comprise 11 to 20 of the consecutive amino acid residues immediately N-terminal to the first systeine of the core sequence of said VRP subunit.
- 13. The truncated VRP subunit according to claim 1, further comprising at the N-terminus of said truncated VRP subunit, the first one or two amino acid residues of the mature non-truncated VRP subunit.
- 14. A truncated VRP comprising two VRP subunits of claim 13.
- 15. A truncated VRP comprising two VRP subunits of claim 25 l, wherein said two VRP subunits have the same amino acid sequence.
  - 10. A trumpated MSO heternähmen emmprisan;
  - a furst, substitut numpersons a réconsanés VPS aubunus of L'alaim 1, and
    - a second subunit comprising a subunit selected from the group consisting of VRP subunits, and a truncated VRP subunit it claim 1, wherein said second subunit has a different aming and appears than said infat Subunit.

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- 17. A nucleic acid molecule coding for a truncated VRP subunit of thaim 1.
- 5 18. The nucleic acid molecule of claim 17 wherein the nucleic acid molecule is a DNA molecule.
  - 19. The nucleus acid molecule of claim 17 wherein the nucleus acid molecule is an RNA molecule.
- 20. A recombinant DNA vector comprising the nucleic acid molecule of claim 17.
- 21. A recombinant DNA expression vector comprising a 15 nucleic acid molecule of claim 17.
  - 22. The recombinant DNA expression vector of claim 21 wherein said nucleic acid molecule is operably linked at the 5' end of said nucleic acid molecule to a DNA sequence that codes for a signal peptide.
  - 23. The recombinant DNA expression vector of claim 22 wherein said signal peptide is selected from the group consisting of VEGF signal peptide, VEGF-B signal peptide, VRF-2 signal peptide, VEGF-C signal peptide, VEGF-3 signal peptide, and PlGF signal peptide.
  - 24. The recombinant INA expression vector of claim IN wherein said gives reprise the end, that from the most consisting is powered .PP-1 dignal peptide, and powered .RP-1 dignal peptide.
  - 27. The recombinant DNA expression vector of claim 22 where.s.s.s.signil pentile is VESF-F signal pentile.

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- it. The recombinant DNA expression vector of claim 12 wherein said DNA sequence coding for said signal peptide is operably linked at the 3' end of said DNA sequence to DNA coding for the first amine acid residue of the mature non-truncated VRP subunit and wherein the 3' end of said DNA coding for said residue is operably linked to said nucleic acid molecule coding for said truncated VRP subunit.
- 27. The recombinant DNA expression vector of claim 22 wherein said DNA sequence coding for said signal peptide is operably linked at the 3' end of said DNA sequence to DNA coding for the first two amino acid residues of the mature non-truncated VRP subunits and wherein the 3' end of said DNA coding for said two residues is operably linked to said nucleic acid molecule coding for said truncated VRP subunit.
  - 28. The recombinant DNA expression vector of claim 22 wherein said nucleic acid molecule is operably linked to control sequences operable in a host cell transformed with said vector.
  - 29. A transformed or transfected host cell comprising the recombinant DNA expression vector of claim 21.
- 25 31. A transformed or transfected host dell comprising the recombinant DNA expression vector of claim 22.
  - II. A transformed of transferred host sell temprising the section and EMA expression section is slaim 16.
  - 33. A delivery vector comprising a nucleic acid molecule of planm 17.

- 33. A delivery vector of claim 32, wherein said delivery vector is a viral delivery vector.
- 34. An adenovirus vector comprising the nucleic acid 5 molecule of claim 17.
  - 35. The adenovirus vector of claim 34 wherein said nucleic acid molecule is operably linked at the 5' end of said nucleic acid molecule to a DNA sequence that codes for a signal peptide.
  - 36. The adenovirus vector of claim 35 wherein said signal peptide is selected from the group consisting of VEGF signal peptide, VEGF-B signal peptide, VRF-2 signal peptide, VEGF-C signal peptide, and PIGF signal peptide.
  - 37. The adenovirus vector of claim 35 wherein said signal peptide is selected from the group consisting of poxvirus ORF-1 signal peptide, and poxvirus ORF-2 signal peptide.
  - 38. The adenovirus vector of claim 35 wherein said signal peptide is VEGF-B signal peptide.
- 39. The adenovirus vector of claim 35 wherein said DNA sequence coding for said signal peptide is operably linked at the 3' end of said DNA sequence to DNA coding for the first amino acid residue of the mature non-truncated VRP subunit, and wherein the 3' end of said CNA coding for daid residue is periply linked to the acciding tor said of companies VEP cucunit.

- 40. A filtered injectable adenovirus vector preparation, comprising: a recombinant adenoviral vector, said vector containing no wild-type virus and comprising:
- a partial adenoviral sequence from which the E1A/E1E genes have been deleted, and
  - a transgene coding for a truncated VRP subunit of claim 1, driven by a promoter flanked by the partial adenoviral sequence; and
    - a pharmaceutically acceptable carrier.

- 41. The preparation of claim 40 wherein said adenovirus vector has been filtered through a 30 micron filter.
- 43. The injectable adenoviral vector preparation according to plaim 40 wherein said promoter is selected from the group consisting of a CMV promoter, a ventricular myocyte-specific promoter, and a myosin heavy chain promoter.
- 43. A method of producing a truncated VRP polypeptide comprising growing, under suitable conditions, a host cell transformed or transfected with the recombinant ENA expression vector of claim II in a manner allowing expression of said polypeptide, and isolating said polypeptide from the host cell.
- 25 44. A pharmaceutical composition comprising a VRP comprising at least one truncated VRP subunit of claim 1, in a suitable carrier.
- 41. A cather to stimulating blind vessel formation 3. Comprising semiconstening to a patient a pharmacountral composition comprising a truncated VWF comprising at least one truncated VRF subunit of claim 1, in a suitable currier.

- 46. A method of stimulating endothelial cell growth or call migration in vitre comprising treating said endothelial cells with a truncated VRP comprising at least one truncated VRP subunit of claim 1, in a suitable carrier.
- 47. A method of treating a patient suffering from a heart tisease comprising administering to said patient a nucleic acid molecule coding for at least one truncated VRP subunit of claim 1, said nucleic acid molecule capable of expressing the truncated VRP subunit in said patient.
- 48. A method of stimulating angiogenesis in a patient comprising administering a therapeutically effective amount of a pharmaceutical composition comprising a truncated VRP comprising at least one truncated VRP subunit of claim 1, in a suitable carrier.
  - 43. The method of claim 43 further comprising a therapeutically suitable delivery system for said pharmaceutical composition.
    - 50. The method of claim 48 further comprising administering a potentiating agent that potentiates the angiogenic effect of said truncated VRP.
    - 81. The method of claim 50, wherein said potentiating agent is an anguagenic FGF.
    - 50. The method of claim fit, wherein said potentiating among is selected from the group constating of FGF-1, FGF-0, FGF-4, FGF-5, and FGF-6.
    - 31. A phasmaceutical composition comprising a truncated VFT scomprising at loast one truncated VPP cubunit of claim 1,

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and one or more potentiating agents in a pharmaceutically acceptable carrier.

- 54. The pharmaceutical composition of claim 53 wherein 5 said potentiating agent is an angiogenic FGF.
  - 55. The pharmaceutical composition of claim 54, wherein said potentiating agent is selected from the group consisting of FSF-1, FGF-2, FGF-4, FGF-5, and FGF-6, in a pharmaceutically acceptable carrier.
  - 56. A method of treating a patient suffering from an ischemic condition comprising administering a therapeutic amount of a pharmaceutical composition comprising a truncated VRP comprising at least one truncated VRP subunit of claim 1, in a suitable carrier.
- 57. The method of claim 56 further comprising administering an agent that potentiates the therapeutic effect of said truncated VRP subunit.
  - 58. The method of claim 57 wherein said potentiating agent is selected from the group consisting of FGF-1, FGF-2, FGF-4, FGF-5, and FGF-6.
  - 59. The method of claim 56 wherein said ischemic condition is selected from the group consisting of: cardiac infection, carenia correctly consents, thronic lower limb inchemia, stroke, and perioderal vaccular alsease.
  - 60. A method for treating applient suffering from a would comprising administering a frequencial amount of a pharmateutical composition comprising a truncated UFF

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comprising at least one truncated VRP subunit, according to claim 1, in a suitable corrier.

- 61. A method of increasing vascular permeability comprising administering a therapeutic amount of a pharmaceutical composition comprising a truncated VRP comprising at least one truncated VRP subunit according to claim 1, in a suitable carrier.
- 62. A method of stimulating angiogenesis in a patient comprising delivering a delivery vector to the myocardium of the patient by intracordnary injection directly into one or both coronary arteries, said vector comprising a nucleic acid molecule coding for at least one truncated VRP subunit according to claim 1, wherein said vector is capable of expressing the truncated VRP subunit in the myocardium.
  - 63. The method of claim 62, wherein said delivery vector is a replication-deficient adenovirus vector.
  - 64. A method for stimulating coronary collateral vessel development in a patient having myocardial ischemia, comprising delivering a delivery vector to the myocardium of the patient by intracoronary injection directly into one or both coronary arteries, said vector comprising a nucleic acid molecule coding for a truncated VRP subunit and capable of empressing the truncated VRP subunit in the myocardium, thereby promoting already tellateral reservices.
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delivering a delivery vector to the peripheral vascular system of the patient by intra-temoral artery injection directly into one or both femoral arteries, said vector comprising a transgene coding for a truncated VRP subunit, and capable of expressing the truncated VRP subunit in the peripheral vascular system, thereby promoting peripheral vascular development.

67. The method of claim 66, wherein said delivery vector is a replication-deficient adenovirus vector.

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Figure 2a VEGF-B

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## Figure 2b VRF-2

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Figure 20 VEGF-C

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